



Chromosomal Abnormalities and Putative Susceptibility Genes in Autism Spectrum Disorders

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Acknowledgments.....	2
List of included papers.....	3
List of abbreviations.....	4
English summary	10
Dansk resumé.....	11
1 Introduction	12
1.1 Classification of Autism Spectrum Disorders (ASDs)	12
1.1.1 Childhood autism	13
1.1.2 Atypical autism.....	14
1.1.3 Asperger's syndrome.....	14
1.2 Genetics	14
1.2.1 Family and Twin studies	14
1.2.2 Comorbid disorders	15
1.2.3 Possible genetic inheritance models	16
1.2.3.1 Single gene (major locus) model.....	16
1.2.3.2 Poly- or multigenic inheritance models	17
1.2.3.3 Interplay between genetic and environmental factors	17
1.2.3.4 X-linkage	18
1.3 Biological changes identified in ASDs	19
1.4 Strategies to identify susceptibility genes for ASDs.....	20
1.4.1 Linkage analyses	20
1.4.2 Mapping of chromosomal rearrangements	21
1.4.3 ASDs associated with disorders with known genetic origin	22
1.4.4 Candidate gene approach	25
1.5 Proposed susceptibility genes for ASDs and their functions	25
2 Objectives of present study	28
3 Paper I.....	30
4 Paper II.....	48
5 Paper III.....	55
6 Paper IV	66
7 Paper V	83
8 Discussion.....	95
9 Conclusions and perspectives.....	100
10 Appendix A	102
11 Appendix B	103
12 Reference List	104

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LIST OF INCLUDED PAPERS

- I. **Gilling M**, Briciet Lauritsen M, Møller M, Henriksen KF, Vicente A, Oliveira G, de Aguiar CC, Eiberg H, Andersen PS, Mors O, Brøndum-Nielsen K, Cotterill R, Lundsteen C, Ropers H-H, Ullmann R, Bache I, Tümer Z, Tommerup N. *Identification of several putative susceptibility genes for childhood autism*. In preparation.
- II. **Gilling M**, Ullmann R, Boonen SE, Brøndum-Nielsen K, Kalscheuer V, Tommerup N, Tümer Z. *KCNQ3 is disrupted in a male patient with childhood autism*. In preparation.
- III. **Gilling M**, Bache I, Niebuhr E, Ullmann R, Tümer Z, Tommerup N. *FGF2 antisense gene disrupted in a male with autism spectrum disorder*. In preparation.
- IV. **Gilling M**, Ullmann R, Kristoffersson U, Møller M, Henriksen KF, Bugge M, Kalscheuer VM, Lundsteen C, Tümer Z, Tommerup N. *Indication of abnormal synapse formation in male twins with autism spectrum disorder*. In preparation.
- V. **Gilling M**, Dullinger JS, Gesk S, Metzke-Heidemann S, Siebert R, Meyer T, Brøndum-Nielsen K, Tommerup N, Ropers H-H, Tümer Z, Kalscheuer VM, Thomas NS. *Breakpoint cloning and haplotype analysis indicate a single origin of the common Inv(10)(p11.2q21.2) mutation among northern Europeans*. Am J Hum Genet 2006 May;78(5):878-83.

LIST OF ABBREVIATIONS

ADAM23	A disintegrin and metalloproteinase domain 23 protein
ADHD	Attention deficit and hyperactivity disorder
ADI-R	Autism diagnostic interview-revised
ADOS	Autism diagnostic observation schedule
ARX	Aristaless related homeobox
ASDs	Autism spectrum disorders
ATOH1	Homolog of atonal 1
AUTS2	Susceptibility to autism 2
AVPR1A	Arginine vasopressin receptor 1A
BAC	Bacterial artificial chromosome
BCL2	B-cell CLL/lymphoma 2
BFNC	Benign familial neonatal convulsions
BRUNOL4	Bruno-like 4 RNA binding protein
BAALC	Brain and acute leukaemia gene
c18orf10	Chromosome 18 open reading frame 10
c18orf37	Chromosome 18 open reading frame 37
CDH9	Cadherin 9
cDNA	Complementary DNA
CGH	Comparative genome hybridization
CHRNA1	Cholinergic receptor, nicotinic, alpha 1
CJ070	Zinc finger CDGSH-type domain 1
CNV	Copy number variations
CREB1	cAMP responsive element binding protein 1
CYLN2	Cytoplasmic linker 2
dATP	2'-deoxyadenosine 5'-triphosphate
DCAMKL1	Doublecortin and CaM kinase-like 1
DEPC	Diethylpyrocarbonate
DHCR7	7-dehydrocholesterol reductase
DHPLC	Denaturing high performance liquid chromatography
DLG1	Homolog of disc large 1
DLG3	Homolog of disc large 3
DSCAM	Down syndrome cell adhesion molecule isoform

DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, fourth revision
DTNA	Dystrobrevin alpha
DZ	Dizygotic
ELAV	Embryonic lethal abnormal visual system
EN2	Engrailed homolog 2
ERBB4	V-erb-a erythroblastic leukaemia viral oncogene
ERK	Extracellular signal regulated kinase
EST	Expressed sequence tag
FAT	Human homolog of the Drosophila fat tumour suppressor gene
FGF12	Fibroblast growth factor 12
FGF2	Fibroblast growth factor 2
FGF2AS	FGF2 antisense
FGF5	Fibroblast growth factor 5
FGFR	Fibroblast Growth Factor receptors
FHOD3	Formin homology domain 3
FISH	Fluorescence In Situ Hybridization
FMR1	Fragile X mental retardation 1
FMRP	Fragile X Mental Retardation Protein
FTSJ1	FtsJ homolog 1
FZD5	Frizzled 5
FZD9	Frizzled 9
GABA	Gamma-aminobutyric acid
GABRA4	GABA A receptor alpha subunit 4
GABRA5	GABA A receptor alpha subunit 5
GABRB3	GABA A receptor beta subunit
GABRG1	GABA A receptor gamma subunit 1
GABRG3	GABA A receptor gamma subunit 3
GALNT1	Polypeptide N-acetylgalactosaminyltransferase
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GLRA3	Glycine receptor, alpha 3
GLRB	Glycine receptor, beta
GPR1	G protein-coupled receptor 1

GRIA2	Glutamate receptor, ionotropic, AMPA 2
GRID2	Glutamate receptor, ionotropic, delta 2
GRIK2	Glutamate receptor 6
GRIPAP	GRIP1 associated protein 1
GRPR	Gastrin-releasing peptide receptor
GTF2I	General transcription factor II
GTF2IRD1	GTF2I repeat domain containing 1
GTP	Guanosine triphosphate
HDAC6	Histone deacetylase 6
Hoxa1	Homeobox A1
IBD	Identical by descent
ICD-10	International Classification of Diseases, tenth revision
IFNA13	Interferon alpha family gene 13
IFNA2	Interferon alpha family gene 2
IMGSAC	International molecular genetic study of autism consortium
IPMK	Inositol polyphosphate multikinase
IQ	Intelligence quotient
KCNMA1	Large conductance calcium-activated potassium
KCNQ3	Potassium voltage-gated channel KQT-like protein 3
KCNQ5	Potassium voltage-gated channel KQT-like protein 5
KLF7	Kruppel-like factor 7
LCRs	Low copy number repeats
LIMK1	LIM domain kinase 1
LOD	Logarithm of odds
LTP	Long term potentiation
LTR	Long terminal repeats
MAB21L1	Mab-21-like protein 1
MAGUK	Membrane-associated guanylate kinase homolog
MAOA	Monoamine oxidase A
MAP2	Microtubule-associated protein 2
MAPK	Mitogen Activated Protein Kinase
MAPK10	Mitogen-activated protein kinase 10
MAPRE2	Microtubule-associated protein

MCN	Mendelian Cytogenetics Network
MCNdb	Mendelian Cytogenetics Network Database
MECP2	Methyl CpG binding protein 2
MMR	Measles, mumps, rubella
MOCOS	Molybdenum cofactor sulfurase
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MTNR1A	Melatonin receptor 1A
MZ	Monozygotic
NBEA	Neurobeachin
NCBI	National centre for biotechnology information
NDN	Necdin
NF1	Neurofibromatosis type one
NLGN3	Neuroligin 3
NLGN4	Neuroligin 4
NPY1R	Neuropeptide Y receptor Y1
NPY5R	Neuropeptide Y receptor Y5
NRP2	Neuropilin 2
NUDT6	Nudix-type motif 6
OCD	Obsessive compulsive disorder
PATRRs	Palindromic AT-rich repeat sequences
PBS	Phosphor buffer saline
PCP4	Purkinje cell protein 4
PCR	Polymerase chain reaction
PDD	Pervasive developmental disorders
PPVT	Peabody picture vocabulary test
PQBP1	Polyglutamine binding protein 1
PRSS21	Serine protease 21
PRSS33	Serine protease 33
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
PSD97	Postsynaptic density protein 97
PTEN	Phosphatase and tensin homolog

Q-PCR	Quantitative PCR analysis
RAB GAP	Rab GTPase activating protein
RAB-GDI	Rab GDP-dissociation inhibitor
RAB-GEF	Rab Guanine nucleotide exchange factor
RIM2	Rab3A-interacting molecule 2
RP11/RPCI11	Roswell park cancer institute
SAP102	Synapse associated protein 102
SAP97	Synapse associated protein 97
SCC	Saccharopine dehydrogenase
SCG2	Secretogranin II precursor
SINE	Short interspersed nuclear elements
SLC39A6	Solute carrier family 39
SMAD9	MAD, mothers against decapentaplegic homolog 9
SNARE	Soluble NSF attachment receptor
SNCA	Alpha-synuclein isoform
SNP	Single nucleotide polymorphism
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SNURF	SNRPN upstream reading frame protein
SPATA5	Spermatogenesis associated factor SPAF5
SST	Somatostatin preproprotein
STATIP1	Signal transducer and activator of transcription 1
STX1A	Syntaxin 1A
SYNI	Synapsin I
SYP	Synaptophysin
SYT1	Synaptotagmin
TDO2	Tryptophan 2,3-dioxygenase
TFAM	Transcription factor A, mitochondrial
Tm	Melting temperature
tRNA	Transfer RNA
TS	Tuberous sclerosis
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
UBE2D1	Ubiquitin-conjugating enzyme E2D

UBE3A	Ubiquitin protein ligase 3A
UCSC	University of California Santa Cruz
UCSs	Ultra conserved sequences
VCX3A	Variable charge, X-linked 3A
WAIS-R	Wechsler Adult Intelligence Scale-Revised
WHO	World Health Organization
XLMR	X-linked Mental Retardation
ZBTB7C	Zinc finger and BTB containing 7C gene
ZNF24	Zinc finger protein 24
ZNF271	Zinc finger protein 271

ENGLISH SUMMARY

Autism spectrum disorders (ASDs) is a heterogeneous group of neurodevelopmental disorders with a significant genetic component as shown by family and twin studies. However, only a few genes have repeatedly been shown to be involved in the development of ASDs. The aim of this study has been to identify possible ASD susceptibility genes.

Genome screens in ASD patients suggest possible susceptibility gene regions on almost every chromosome. We identified four ASD patients with chromosomal rearrangements, two of which were familial rearrangements involving one of these putative susceptibility gene regions and two were *de novo* rearrangements. We characterised all chromosomal breakpoints at the molecular level with **Fluorescence in situ hybridization (FISH)** and Southern blot analysis when necessary. In addition, **Bacterial artificial chromosome (BAC) array-CGH (Comparative genome hybridization)** was performed for all four patients. By combination of these methods we identified several putative susceptibility genes for ASDs. Expression patterns were established for several of these genes by **Quantitative PCR (Q-PCR)** or in situ hybridization and one gene was sequenced in 157 ASD patients. Our results support a complex genetic basis of ASDs and that detailed molecular dissection of patients with inherited as well as *de novo* chromosomal rearrangements may reveal information about susceptibility genes for ASDs. In addition, two of the candidate susceptibility genes identified provide a potential link between a genetic predisposition and an environmental factor (stress) that in a mouse model system result in a male specific effect. Accordingly, these two autosomal genes are candidates for the male preponderance in ASDs.

DANSK RESUMÉ

Autisme spektrum forstyrrelser (ASDs) er en heterogen gruppe af forstyrrelser der er forårsaget af defekter i den neuronale udvikling. Tvillinge- og familiestudier har tydeligt vist, at genetiske faktorer spiller en stor rolle for udviklingen af disse forstyrrelser, men indtil nu er der kun identificeret ganske få gener, der i mere end ét tilfælde har været involveret i udviklingen af ASDs. Formålet med dette projekt har været at identificere kandidatgener, der øger risikoen for at udvikle ASDs.

Ved hjælp af genomskanninger af grupper af ASD patienter er der blevet identificeret områder på næsten alle kromosomer, der kan indeholde mulige kandidatgener for ASDs. Vi identificerede fire ASD-patienter, der samtidig havde et eller flere kromosomale rearrangementer. To af disse patienter havde arvet rearrangementer hvor mindst ét kromosombrud var inden for et kromosomområde, der tidligere var identificeret som muligt kandidatgen-område. De sidste to ASD patienter havde nyopståede rearrangementer (*de novo*). Vi karakteriserede alle kromosomale brudpunkter med **fluorescens in situ hybridisering (FISH)** og herefter med Southern blot når det var nødvendigt. Herudover blev alle fire patienter undersøgt ved hjælp af **BAC array-CGH (komparativ genom hybridisering)**. Ved at kombinere disse metoder kunne vi identificere adskillige mulige kandidatgener for ASDs. For flere af disse gener brugte vi metoderne **kvantitativ PCR (Q-PCR)** eller in situ hybridisering til at undersøge hvilke væv de var udtrykt i. Vi sekventerede også ét af disse gener i 157 ASD patienter. Vores resultater underbygger tidligere rapporter om at det genetiske nedarvningsmønster for ASDs er komplekst og viser endvidere, at man ved at karakterisere både nedarvede og nyopståede kromosomale rearrangementer kan identificere mulige kandidatgener for ASDs. Herudover etablerer to af de identificerede gener en mulig sammenhæng imellem en genetisk prædisposition og en miljømæssig faktor (stress), der i musemodeller har vist sig at resultere i en effekt specifikt i mænd. Disse to autosomale gener kan derfor være medvirkende til at skabe den store overvægt af mænd med ASDs.

1 INTRODUCTION

Childhood autism was first recognized as a psychiatric disorder distinguishable from mental retardation by Leo Kanner in 1943 [1, 2]. One year later, Hans Asperger described patients with resembling clinical manifestations [2]. Even though both Kanner and Asperger suggested a neuropathological origin for autism, deficiencies in parenting and “refrigerator mothers” were thought to be the cause of the disorder through the 1950s [1, 2]. This hypothesis has for long been proved wrong by twin and family studies that demonstrate a robust genetic foundation for childhood autism and the broader autism spectrum of disorders. Nevertheless, only very few susceptibility genes have been identified for these disorders.

1.1 Classification of Autism Spectrum Disorders (ASDs)

For decades, little agreement has existed on the relative emphasis on phenomenology and aetiology in the classification of mental disorders [3]. To ensure a worldwide agreement on diagnostic criteria, the “**ICD-10 (International Classification of Diseases, tenth revision)** Classification of Mental and Behavioural Disorders” was elaborated within the framework of the **World Health Organization (WHO)** [4]. In parallel, the “**Diagnostic and Statistical Manual of Mental Disorders, fourth revision (DSM-IV)**” was developed by the American Psychiatric Association and effort has been made to unify these two classification systems to avoid discrepancies [3]. DSM-IV is used in many countries besides America, however, in Denmark ICD-10 is used.

Childhood autism, atypical autism, Asperger’s disorder, Rett syndrome and childhood disintegrative disorder are differential diagnoses of **pervasive developmental disorders (PDDs)** that are characterized by severe impairments in reciprocal social interaction and verbal and nonverbal communication as well as the presence of limited, stereotyped behaviour, interests and activities [3, 4]. These qualitative impairments are reflected in all situations, but the severity can vary greatly between individuals [4]. Childhood autism, atypical autism and Asperger’s disorder are often in unity referred to as **autism spectrum disorders (ASDs)**. The prevalence of ASDs is estimated to be between 27,5 and 116,1 in 10.000 [5-7] with a male to female ratio of four to one [2].

The diagnostic criteria for childhood autism have changed over time and in addition, concepts like ASDs are not being used consistently throughout the literature. This sometimes makes it difficult to categorize old findings in up-to-date clinical concepts. In this introduction I have consistently tried to categorize the original clinical descriptions into either of the groups “childhood autism” or the broader “ASDs”.

1.1.1 Childhood autism

Childhood autism is also referred to as infantile autism, Kanner’s autism or simply autism [3, 4]. Approximately 1/3 (10-38,9 of 10.000) of all individuals diagnosed within the ASD spectrum have the “childhood autism” diagnosis [5, 6]. Some symptoms and signs of childhood autism can be present from birth but most children will not fulfil the criteria for a childhood autism diagnosis before the age of 18-24 months where deficits in developing phrase speech and participating in interactive play become apparent [2-4]. However, by definition, the onset of childhood autism is before three years of age [2-4].

The impairment in reciprocal social interaction is reflected in many ways. These children often have little or no interest in establishing friendships and in addition do not understand the standards of social interaction or the needs of others [2-4]. Accordingly, most of these children fail to develop relationships appropriate to developmental level [2-4]. Moreover, abnormal use of eye contact, facial expression and gestures are common observations [2-4].

It is estimated that between 1/3 and 1/2 of children with childhood autism never develop an actual language [2, 8]. In the remaining individuals, development of speech is delayed and the pitch, rhythm or stress may be abnormal and grammatical structures are immature and repetitive [2-4]. Moreover, individuals with speech may have difficulties in initiating or sustaining a conversation [3, 4].

The third diagnostic criterion that defines childhood autism is “restricted, repetitive, and stereotyped patterns of behaviour, interests, and activities”[3]. This includes inflexibility to deviate from routines and rituals, stereotyped and repetitive motor mannerisms (clapping), overly focused interests (remembering bus timetables, soccer statistics etc.) and a persistent preoccupation with inanimate objects [3, 4]. In some cases, abnormal body posture or body movements (walking on tip toe, odd hand movements) are observed [3, 4].

In addition to the characteristics of childhood autism described above, several other features are commonly found in these individuals [3, 4]. Their reaction to sensory stimuli may be abnormal in the form of oversensitivity to sounds or touch or a high pain-threshold [3, 4]. Abnormal eating or sleeping habits can be seen as well as unusual emotional reactions such as fear in response to harmless objects and a lack of fear in response to real dangers and giggling or weeping for no apparent reason [3, 4]. Moreover, self-injurious behaviours may be present [3, 4].

1.1.2 Atypical autism

Atypical autism is distinguished from childhood autism either by having a later onset or by partial fulfilment of the three key diagnostic criteria (impairment in reciprocal social interaction and communication in addition to repetitive and restricted behaviour and interests) [4].

1.1.3 Asperger's syndrome

Individuals with Asperger's syndrome manifest abnormal social interaction and restricted, repetitive patterns of behaviour and interests as described for childhood autism [3, 4]. However, contrary to childhood autism, there is no significant delay in language and cognitive development [3, 4]. Developmental delay in motor function or motor clumsiness is nonetheless often observed [3, 4]. Asperger's syndrome is eight times as frequent in males compared to females [4].

1.2 Genetics

1.2.1 Family and Twin studies

A genetic aetiology for childhood autism was first suggested from the observation that the risk of having a second child with childhood autism (the recurrence rate) was approximately 3% [9], which is up to 10 times as high as in the general population. Subsequently, twin studies clearly showed that the genetic contribution to the development of childhood autism was significant. When comparing the concordance rate of *monozygotic (MZ)* versus *dizygotic (DZ)* twin pairs diagnosed with childhood autism the relative magnitude of genetic and environmental influence on the development of the disorders can be estimated: the higher the MZ versus the DZ concordance rate, the more important is the genetic contribution. Three twin studies have been carried out for same-

sex twins with childhood autism (reviewed in [2]). It is important that the twins are of same sex considering the significant male preponderance in this disorder. At least one twin in each pair was diagnosed with childhood autism. Only 66 twin pairs in total were involved in these studies, 36 MZ pairs and 30 DZ pairs. On average a MZ concordance rate of 70% and a DZ concordance rate of 0% were reported. When broadening the phenotype in co-twins to include milder cognitive or social deficits the MZ concordance rate increased to 82% and the DZ concordance rate to 10%. The low DZ concordance rate observed when using the strict phenotype criteria (0%) is suggested to be an outcome of the few DZ twin pairs in the studies since it is expected to be identical to the recurrence risk (3%) [2]. The considerably higher MZ versus DZ concordance rate indicates that childhood autism and the associated milder phenotypes observed in MZ twins are highly heritable [2]. This is further substantiated by the observation that Asperger's syndrome clusters in families [10] and the recognition of one, two or three of the conceptually same traits as those defining childhood autism (impaired social and communication skills, preference for routines and difficulty with change) in close family members (parents, siblings, first cousins) of ASD children than of controls [2, 11]. These milder traits have become known as "the broader autism phenotype" and are usually not associated with difficulties in functioning and might even be associated with high achievement [2]. "The broader autism phenotype" is more commonly observed in male compared to female relatives [11]. Moreover, delayed onset of speech and difficulty with reading are also common findings in family members of ASD patients [2, 12] in addition to bipolar disorder [10, 13], major depression [14], schizophrenia [10] and anxiety disorders [15]. This suggests an overlap in either diagnostic criteria or genetic susceptibility factors for at least some of these disorders and ASDs.

1.2.2 Comorbid disorders

In approximately 3/4 of children with childhood autism a comorbid diagnosis of mental retardation of varying degree is made^A [3]. In addition, 5 - 38,3% of ASD patients develop epilepsy [16, 17]. The major risk factor for developing epilepsy is severe mental retardation with or without motor deficits [16, 17]. Moreover, 22% of ASD patients present tic disorders: 11% have Gilles de la Tourette syndrome whereas 11% have chronic motor tics [18].

^A DSM-IV: profound mental retardation: IQ<20; severe mental retardation: 20≤IQ≤34; moderate mental retardation: 35≤IQ≤49; mild mental retardation: 50≤IQ≤69 [3].

Individuals with ASDs are frequently reported to have abnormal emotional and behavioural reactions such as hyperactivity, short attention span, impulsivity, aggressiveness, self-injurious behaviours and temper tantrums [3]. In line with this, Leyfer et al found at least one comorbid psychiatric disorder in 72% of children diagnosed with childhood autism [19]. Of these, 44% had comorbid phobia, 37% had **obsessive compulsive disorder (OCD)** and 25% had major depression [19]. In addition, Stahlberg et al. found that 38% of ASD patients had comorbid **attention deficit and hyperactivity disorder (ADHD)** whereas 7% had bipolar disorder and 7,8% had schizophrenia [20]. The high incidence of comorbid psychiatric disorders in individuals with ASDs implicate, that ASD patients should be tested for additional psychiatric disorders since the symptoms of several of these can be relieved by drugs [19].

The finding of comorbid “brain disorders” in a considerable proportion of ASD individuals suggests diagnostic overlap and/or a common genetic aetiology for at least some of these disorders.

1.2.3 Possible genetic inheritance models

In approximately 5-10% of individuals with ASDs an associated genetic disorder (described later), chromosomal rearrangement or environmental agent has been inferred as the cause of the disorder [21]. This renders the remaining approximately 90% of ASD cases idiopathic (of unknown cause) [21]. When looking at the actual findings in family and twin studies, information about the possible inheritance models emerge.

1.2.3.1 Single gene (major locus) model

If childhood autism was inherited as an autosomal recessive or fully penetrant dominant Mendelian disorder, a sibling recurrence risk of 25% and 50%, respectively, would be expected. Instead a recurrence risk of 3% is observed [9]. This argues against a Mendelian inheritance pattern for at least the majority of childhood autism cases. However, approximately 3% of ASDs are associated with chromosomal abnormalities (translocations, inversions, deletions, duplications and supernumerary markers) [21] and these abnormalities are in most cases suggested to be the primary cause of the phenotype, albeit additional genes might modulate the phenotypic outcome.

1.2.3.2 Poly- or multigenic inheritance models

The occurrence pattern in pedigrees suggests the most likely inheritance model for ASDs to involve several epistatic genes [22]. The suggested number of genes involved ranges from two to 10 with the most likely being three epistatic genes [22]. Hence, it is suggested that ASDs occur when a child inherits approximately three genes from the parents that each contribute to the phenotype [2]. However, whether these genes are always the same or whether several combinations of genes from a larger pool of susceptibility genes can cause ASDs is not specified by this model [2]. This model suggests that the inherited genes are all part of the same biological pathway and therefore with additive effect result in the full ASD phenotype [22]. An alternative multigenic inheritance model arises by considering the ASD phenotype as a combination of elements (social reticence, repetitive behaviour, impaired communication skills) that are inherited separately [12, 22]. This model does not fit data well when assuming a single locus for each phenotypical element [22], but it might be possible that each of these elements is also polygenic. In poly- or multigenic models like these, the male preponderance in ASDs and the broader autism phenotype is explained as a lower threshold in males compared to females [11]. Accordingly, female probands require a relatively higher number of risk factors to develop ASDs and an excess of affected family members of female probands is therefore expected [11]. However, there is no difference in familial loading, or variation in severity and type of expression of the broader autism phenotype in relatives of male and female probands, which implies that a simple sex-limited additive genetic multigenic threshold model can not explain the observed inheritance patterns [11].

1.2.3.3 Interplay between genetic and environmental factors

A concordance rate of 100% in MZ twins is expected for a purely genetic disorder. The slightly lower observed concordance rate (70-82%) in ASDs is therefore consistent with the involvement of environmental factors in the development of ASDs [11, 23].

In utero exposure to ethanol, thalidomide, valproic acid or misoprostol early within the first trimester of pregnancy increases the risk of developing ASDs [24-27]. The pathological mechanisms underlying these associations are unknown but it has been suggested that valproic acid dysregulates retinoic acid, which subsequently leads to altered gene expression of **homeobox A1 (Hoxa1)** among other genes [24, 26].

In addition, a number of studies indicate that dysregulation or dysfunction of the immune system might be involved in the development of ASDs in some individuals [28]. The pathological effects have been suggested to be mediated either by auto-antibodies or by an ineffective immune response to pathogen challenges [28] but infection of the fetal CNS with viruses (rubella and measles) during critical times of development can also lead to ASDs [28]. In addition, animal models suggest that the maternal immune response to infections during pregnancy can influence brain development in the fetus and thus possibly cause ASDs [28]. Childhood vaccinations, such as the **measles, mumps, rubella (MMR)** vaccine, have also for long been suspected to cause ASDs, but there is no scientific evidence confirming that the vaccinations as such or the mercury preservatives used in some vaccinations are involved in the development of ASDs [29].

Maternal care during development result in animals less emotional and in general better able to respond to the demands of the environment [30]. Since maternal care during development increases innervations of the hippocampus and enhances spatial learning and memory this might be the responsible underlying biological mechanism [30]. On the contrary, pre- and postnatal stress can be maladaptive and lead to impairments in challenging situations later in life [30]. In agreement with this, mothers of children with autism have reported significantly more stressful events during pregnancies than mothers of non-autistic children [31]. The underlying biological mechanisms are not known but experiments in rats suggest how this might happen and how the effect can be male-specific: Chronic stress for 21 days in adult rats that had been exposed to corticosterone late in pregnancy (corresponding to prenatal stress) caused dendritic atrophy of the CA3 pyramidal neurons in male rats but not in female rats [30]. Hence, it is clear that pre- and postnatal stress can influence brain development but whether this leads to increased susceptibility to ASDs and to what extent it is dependent on genetic predisposition is not known.

1.2.3.4 X-linkage

A considerable male preponderance is observed for ASDs. This immediately suggests an X-linked inheritance pattern. In favour of this theory is that some mutations in the X-linked genes causing Rett syndrome (**methyl CpG binding protein 2; MECP2**) [32] and Fragile X syndrome (**fragile X mental retardation 1; FMR1**) [33] as well as in **neuroligin 3 and 4 (NLGN3 and 4)** [34, 35] and **aristaless related homeobox (ARX)** [36] can cause ASDs. However, the father-to-son transmission of ASDs (including the broader

phenotype) together with the lack of significant linkage signals on the X-chromosome implies either that X-linkage explains only a minor proportion of the sex-specific variance [2], that not one or a few X-linked genes are involved but instead numerous different X-linked genes may contribute to the phenotype or that the X-specific factor may be epigenetic in nature [2]. Several studies have implied how this latter suggested factor might work. In general, normal females tend to have better social and communicative skills compared to males and accordingly, ASDs have been suggested to represent an extreme male pattern [2, 37]. In addition, subjects with Turner syndrome (45,X karyotype) had considerably better social skills if their single X-chromosome was of paternal origin compared to those with an X of maternal origin [38]. Hence, the presence of a maternally imprinted locus on the X-chromosome was suggested [38]. According to this “imprinted-X liability threshold model of risk for ASDs”, genetic vulnerability is due primarily to the effects of autosomal genes that are equally inherited from the father and the mother, but females have a higher threshold for expressing the ASD phenotype due to expression of the paternally derived X-linked gene [39]. In such a model females can be affected either due to a skewed X-inactivation pattern or because the imprinted locus on the paternally inherited X-chromosome is damaged or silenced by rearrangements or mutations [39]. This model has however been questioned by the identification of solely paternally imprinted genes on the X-chromosome in mouse [40]. Other mechanisms could be involved in creating the considerable sex-difference: over-expression of maternal-specific X-linked genes might lower the ASD threshold in males but not in normal females due to random X-inactivation [40], genes on the Y-chromosome might lower the ASD threshold in males, or sex-hormones might play part by creating an extreme male brain pattern [37]. None of these theories have to date been able to explain the considerable male preponderance in ASDs, including the lower male threshold for developing ASDs suggested by the multigenic inheritance model.

1.3 Biological changes identified in ASDs

Several neurobiological changes have been identified in individuals within the ASD spectrum; however, none of these are present in all ASD patients.

The brain is often enlarged in children with ASDs and it is suggested that this enlargement occurs predominantly in the postnatal period [41]. Apart from this finding, neuropathological and **MRI (Magnetic Resonance Imaging)** data are equivocal and it is

suggested that this might be due to small sample size, heterogeneity of the disorders or inability to control for confounding variables such as gender, mental retardation and epilepsy [42]. However, the most consistent findings in neuropathological investigations of ASD individuals are: reduced cell size including reduced dendritic arborisation and increased cell packing in hippocampus and amygdala; a decrease in size and number of Purkinje cells in the cerebellum and dysgenesis of the neocortex including high neuronal density and poor lamination [41, 42]. Moreover, disrupted development of multiple neurotransmitter systems (Glutamate, GABA, Acetylcholin, Serotonin, Catecholamines) have been identified in ASD patients and it has accordingly been suggested that ASDs might arise when the balance between excitatory and inhibitory neurotransmitter systems has been skewed [41, 43].

1.4 Strategies to identify susceptibility genes for ASDs

1.4.1 Linkage analyses

The main statistical tool used for analyzing the inheritance of ASDs are segregation analysis [44]. Linkage analyses have been used with success to identify several Mendelian disease genes [45-47]. However, for complex disorders like ASDs there are several problems in employing this strategy [44]. Since the mode of inheritance of ASDs has not been determined it is not possible to provide the system with a genetic model (autosomal recessive, dominant etc.) which is required in parametric analyses [44]. One way to circumvent this problem is to identify families with “near-Mendelian” inheritance patterns [44]. The “near-Mendelian” inheritance pattern in a family might arise because some families have a Mendelian form of the disorder that phenotypically can not be distinguished from the non-Mendelian majority, or it might arise because several susceptibility genes are, by chance, present in the family and the Mendelian segregation of one susceptibility factor tips the balance [44]. Such families are, however rare since the majority of individuals with ASDs do not have children. Another way to circumvent the problem of parametric linkage analysis is to perform non-parametric (without model) linkage analysis [44]. Such methods identify chromosomal segments that are shared more often by affected individuals in a family than would be expected [44]. Due to random segregation, sib pairs share 0, 1 or 2 parental chromosome segments with frequencies $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$, which means that chromosomal regions where sharing is above

the expected frequency might harbour a susceptibility gene for the shared disorder [44]. There are, however, some drawbacks of this method that should be kept in mind. Successful linkage analyses rely on reproducible diagnostic criteria and are sensitive to genetic heterogeneity [44]. Moreover, sib pairs share many chromosomal segments by chance which can lead to false positives and if a susceptibility factor is neither necessary nor sufficient for the development of ASDs then not all affected sib pairs will share a chromosomal segment and linkage might not be established [44]. Nevertheless, genome screens or sib pair analyses like these are among the most frequently used methods for identifying susceptibility genes for ASDs. Regions on almost every chromosome have been suggested to harbour susceptibility genes for ASDs, but only a few of these regions have been replicated by different groups. Results from linkage studies for ASDs are summarized in Appendix A and in [2, 48].

1.4.2 Mapping of chromosomal rearrangements

Chromosomal rearrangements such as translocations, inversions, insertions, deletions and duplications might alter the expression pattern or expression level of one or more genes and hence cause disease. Accordingly, molecular characterization of such changes has pinpointed disease genes for several Mendelian disorders [49, 50] as well as susceptibility genes for complex disorders [51, 52]. Even though chromosomal rearrangements can result in disease this is far from always the case. Therefore, *de novo* chromosomal rearrangements or rearrangements that segregate with a phenotype in a family have preferably been studied.

Approximately 3% of ASD individuals have chromosomal abnormalities [21]. The rearrangements are not distributed evenly across the genome but rather tend to cluster in specific regions [48]. The autosomal abnormalities most frequently reported to be involved in ASD patients are: maternal duplications of the Prader Willi/Angelman syndrome region on 15q11-13 (either tandem repeats or marker chromosome) as well as loci along most of 7q in addition to 22q11.2 and 22q13.3 [48]. Many chromosomal rearrangements in ASD patients have already been examined with molecular techniques such as ***Fluorescence In Situ Hybridization (FISH)***. The putative susceptibility genes for ASDs identified through chromosomal rearrangements are listed in Appendix B.

1.4.3 ASDs associated with disorders with known genetic origin

Rett syndrome, fragile X syndrome, tuberous sclerosis, neurofibromatosis, Angelman and Prader Willi syndrome are medical disorders with known genetic aetiology that are all frequently associated with ASDs. Deciphering the molecular mechanisms behind these associated disorders might shed light on the biological pathways involved in the development of ASDs and thereby reveal additional susceptibility genes.

Rett syndrome is characterized by mental retardation, autistic behaviour and sometimes epilepsy [53]. Mutations in *MECP2* at Xq28 are most often the cause of Rett syndrome but a few mutations have been identified in female patients with isolated ASD phenotypes [53, 54]. The MECP2 protein is involved in transcriptional suppression and localizes to the nuclear compartment as well as postsynapses where it plays a key role in the control of neuronal activity-dependent gene regulation [53, 55]. *Mecp2* deficiency in mice leads to temporal and regional changes in expression of proteins involved in cytoskeletal rearrangement, chromatin modelling, energy metabolism, cell signalling, and neuroprotection [56]. Moreover, Fukuda et al. demonstrated delayed maturation of neurons and reduced **postsynaptic density (PSD)** maturation in *Mecp2*^{-Y} mice suggesting a role of MECP2 in synaptogenesis [53].

Fragile X is the most common inherited form of human mental retardation [57]. The prevalence of childhood autism among individuals with fragile X syndrome is 25-33% whereas the prevalence of fragile X syndrome is estimated to be 2.1% among ASD patients [57]. Fragile X syndrome is primarily caused by expansion of the CGG repeat in the 5' end of the *FMR1* gene at Xq27.3 resulting in hypermethylation and reduced or absent production of the corresponding mRNA [33]. The *FMR1* gene encodes the **Fragile X Mental Retardation Protein (FMRP)**, which is an RNA-binding protein presumably involved in translation [58]. FMRP is abundantly expressed in neurons in the hippocampus and cerebellum [57]. *Fmr1* knock out mice show abnormal dendritic spines [58] and FMRP associates with polyribosomes within and at the base of dendritic spines in wild type neurons [58]. Accordingly, FMRP has been suggested to function as a translational suppressor involved in synaptic plasticity through regulation of local protein synthesis in response to synaptic stimulation [58]. In addition it has been suggested that FMRP may be required for the normal process of maturation and elimination of synapses during cerebral cortical development [59].

The prevalence of childhood autism in individuals with **tuberous sclerosis (TS)** is estimated to be between 16 and 65% and, reversely, the prevalence of TS in individuals diagnosed with childhood autism is 0.4% [57]. Heterozygous mutations in the genes **tuberous sclerosis 1 (TSC1)** (9q34) or **tuberous sclerosis 2 (TSC2)** (16p13) lead to TS, a hamartomatous disorder characterized by benign tumours in brain, kidneys, heart and eyes [60]. Well known neurological manifestations of TS is epilepsy, mental retardation and autism [60]. *TSC2* encodes tuberlin that functions as a **GAP (GTPase-activating protein)** accelerating hydrolysis of GTP to GDP by RAP1 and RAB5 [61, 62]. *TSC1* encodes hamartin that binds tightly to tuberlin *in vivo* [63]. Hamartin and tuberlin participate in a conserved growth-regulating pathway that controls soma size, the density and size of dendritic spines as well as the properties of excitatory synapses in hippocampal pyramidal neurons [60]. In addition, the proteins affect neuronal migration [64].

Neurofibromatosis type one (NF1) is observed in up to 1.4% of individuals with childhood autism [57]. NF1 is an autosomal dominant disease caused by mutations in the *NF1* gene located at 17q11.2 [57]. The disease is characterized by neurofibromas, café-au-lait spots, axillary or groin freckles, predisposition to developing neoplasias and sometimes mental retardation or learning difficulties [57, 65]. *NF1* encodes the tumour suppressor protein neurofibromin [65]. Neurofibromin is a multidomain protein that (like TSC2) functions as a GAP, however for a different GTPase (RAS) [65]. Neurofibromin is involved in the regulation of several intracellular processes including the **ERK (extracellular signal regulated kinase) MAPK (Mitogen Activated Protein Kinase)** signalling cascade, the adenylyl cyclase signalling pathway and cytoskeleton assembly [66]. Also, neurofibromin binds syndecans, which are trans-membrane heparin sulphate proteoglycans that supposedly function as co-receptors in some receptor tyrosin kinase signalling pathways (e.g. FGF) [65]. Moreover, neurofibromin associates with microtubules that are found in high concentrations in axons and dendrites and are among other things important in signal transduction [65]. In addition, *NF1*^{+/-} mice show defects in hippocampal **long term potentiation (LTP)** [66]. All in all this clearly shows that neurofibromin is a versatile protein involved in several different signalling pathways important for memory and learning.

Chromosome 15q11-q13 is the most frequently reported autosomal region involved in chromosomal rearrangements in individuals diagnosed with ASDs [67].

Maternally derived duplications or supernumerary inv dup(15) marker chromosomes are the most common forms of chromosome 15 abnormalities in this patient group [67]. Aberrant imprinting pattern, uniparental disomy as well as deletions in the same region is involved in Prader Willi syndrome and Angelman syndrome that both show some phenotypical overlap with ASDs [57]. Patients with Angelman syndrome have severe mental retardation, show poor motor coordination, seizures and have significant language impairment as seen in ASD patients, whereas patients with Prader Willi syndrome often show aggression, preoccupation with ordering and arranging and resistance to change in daily routines, which are also characteristic features of ASDs [57]. The prevalence of childhood autism in individuals with Prader Willi syndrome is estimated to be 25.3% [57]. Prader Willi syndrome arises due to lack of paternal contribution within this chromosomal region [57]. Approximately 42% of individuals with Angelmann syndrome meet criteria for childhood autism and reversely, 1% of individuals with ASDs have Angelmann syndrome [57]. This syndrome arises due to silencing or disruption of the maternally derived **ubiquitin protein ligase 3A (UBE3A)** [57]. Since ASDs are more frequently observed in patients with Prader Willi syndrome due to maternal uniparental disomy compared to patients where Prader Willi syndrome arose due to a paternal deletion it has been suggested, that overexpression of *UBE3A* confers a risk for developing ASDs [68]. The ubiquitin proteasome pathway is responsible for the degradation of abnormal proteins as well as for the normal turnover of many intracellular proteins [69]. E3 ubiquitin protein ligases (like *UBE3A*) confer specificity to substrate recognition for ubiquitination which subsequently leads to protein degradation by the proteasome [70]. Hence, increased amounts of *UBE3A* due to a maternally inherited duplication most likely result in decreased amounts of the target proteins of which only a few are known. However, a mouse model of Angelman syndrome has shown that reduced amounts of *UBE3A* is associated with a calcium/calmodulin-dependent protein kinase II mediated defect in hippocampal long-term potentiation [70]. In addition to *UBE3A* the non-imprinted genes ***gamma-aminobutyric acid (GABA) A receptor beta subunit (GABRB3)***, ***GABA A receptor alpha subunit (GABRA5)*** and ***GABA A receptor gamma subunit (GABRG3)*** within the Prader Willi/Angelman syndrome region at 15q11-13 have been suggested to be susceptibility genes for ASDs [57].

1.4.4 Candidate gene approach

Putative susceptibility genes for ASDs can also be identified by making educated guesses on the basis of previous findings such as linkage intervals, reported chromosomal rearrangements or neuropathological investigations. This approach was used with success by Jamain et al. who sequenced *NLGN4* located in a region (Xp22.3) that had previously been reported to be deleted in three ASD females [34, 71]. They identified a maternally inherited frameshift mutation in *NLGN4* in a two Swedish brothers, one diagnosed with childhood autism and the other with Asperger's syndrome [34]. In addition they sequenced *NLGN3* at Xq13 and identified a missense mutation in a different Swedish family, where one of the males was diagnosed with childhood autism and the brother with Asperger's syndrome [34]. Isoform expression differences of *NLGN3* and 4 have subsequently been identified in two female ASD patients and additional mutations have been identified in these genes in ASD patients as well as in mentally retarded individuals [35, 72]. Neuroligins are postsynaptic membrane proteins that through their interaction with presynaptic neurexins induce synapse maturation and hence suggests that defect synaptogenesis might be the underlying cause of some forms of ASDs [73].

1.5 Proposed susceptibility genes for ASDs and their functions

Despite ongoing efforts in identifying susceptibility genes for ASDs only a few genes have unambiguously been shown to be associated with ASDs. In the table on the following pages I have listed the putative susceptibility genes for ASDs and the functions of the encoded proteins.

Chr.	Band	Gene	Protein function	Biological pathways	Refs.
2	q31.1	RAPGEF4	Guanine nucleotide exchange factor		[74]
		CHRNA1	Acetylcholin receptor subunit	Neurotransmission	[75]
	q33.3	NRP2	Semaphorin III receptor	Axon guidance	[76]
		GPR1	G protein-coupled receptor		[76]
		ADAM23	Disintegrin and metalloproteinase	Neuronal migration	[76]
		KLF7	Transcription factor	Differentiation of neurons and synaptogenesis	[76]
		CREB1	cAMP responsive element	Synaptic plasticity related to long term memory	[76]
		FZD5	Wnt5A receptor	Regulates neuronal potential	[76]
	q34	MAP2	Microtubule associated protein	Neurite outgrowth	[76]
		ERBB4	Neuregulin receptor	Modulation of synaptic plasticity	[76]
3	q36.1	SCG2	Secretory protein	Neuronal migration	[77]
	q27.3	SST		Migration of cerebellar granule cells	[78]
	q28	FGF12	Fibroblast growth factor 12	Nervous system development/function	[78]
4	p12	GABRA4	GABA receptor subunit	Neurotransmission	[79]
		GABRG1	GABA receptor subunit	Neurotransmission	[80]
	q21.21	FGF5	Fibroblast growth factor 5	Proliferation and/or migration of neurons	[78]
	q21.3	MAPK10	Mitogen activated protein kinase	Stress-induced neuronal apoptosis	[78]
	q22.1	SNCA		Presynaptic activity-dependent regulator of dopamine release	[78]
	q22.1-22.1	GRID2	Glutamate receptor subunit	Neurotransmission	[78]
	q22.2	ATOH1	Proneural gene	Required for granule-cell genesis	[78]
	q22.3	UNC5	Netrin-1 receptor	Cell migration and axon guidance	[78]
	q27-28.1	FGF2	Fibroblast growth factor 2	Neuronal proliferation and protection, synaptogenesis, long term potentiation	Paper III
	q28.1	NUDT6	FGF2 antisense		Paper III
	q32.1	TDO2	Enzyme	Serotonin catabolism	[81]
		GLRB	Glycine receptor subunit	Neurotransmission	[82]
		GRIA2	AMPA receptor subunit	Neurotransmission	[82]
	q32.2	NPY1R	Neuropeptide Y receptor	Affects cognitive function, learning and memory	[82]
		NPY5R	Neuropeptide Y receptor	Affects cognitive function, learning and memory	[82]
	q34.1	GLRA3	Glycine receptor subunit	Neurotransmission	[82]
	q35.2	FAT	Adhesion molecule		Paper I
5	p14.1	CDH9	Adhesion molecule	Synaptogenesis	Paper IV
6	q16.3	GRIK2	Glutamate receptor subunit	Neurotransmission	[83]
7	q11.22	AUTS2			[84]
	q11.23	FZD9	Wnt1 receptor	Critical determinant of hippocampal development	[78]
		STX1A	Post synaptic membrane protein	Vesicle priming and neurotransmitter exocytosis	[78]
		LIMK1	Regulates actin cytoskeleton	Synaptogenesis, Long term potentiation, dendritic spine morphogenesis	[78]
		CYLN2	Links membranous organelles to microtubules	Brain development	[78]
		GTF2IRD1	Transcription factor	Cognitive development	[78, 85]
		GTF2I	Transcription factor		[78, 85]
	q22.1	REELIN	Glycoprotein	Neuronal migration	[86]
	q31.2	RAY1	Multi-transcript system with non-coding RNAs		[87]
8	q36.3	EN2	homeobox transcription factor	Neuron differentiation and guidance of growth cones	[88]
	q22.3	RIM2	Presynaptic membrane protein	Associative memory and learning	Paper IV
		BAALC	Postsynaptic membrane protein	Synaptogenesis	Paper IV
	q24.44	KCNQ3	K ⁺ channel	sensory-motor behaviour, learning and memory	Paper II

Table 1. Chromosome 1-8. Chromosome position, protein function and inferred biological pathway for putative susceptibility genes for ASDs. Chromosome position refers to UCSC march 2006 (hg18). Papers I-IV refers to papers presented in this thesis

Chr.	Band	Gene	Protein function	Biological pathways	Refs.
9	q34.13	TSC1		Inhibition of cell growth, brain development	[57]
10	q22.3	KCNMA1	Ca ²⁺ - and depolarization activated K ⁺ channel	Action potential	[89]
	q23.31	PTEN	Enzyme	Cell cycle arrest and/or apoptosis	[90]
12	q14.2	AVPR1A	Hormone	Induction of social behaviour	[91]
13	q13.2-13.3	NBEA	AKAP family protein		[92, 93]
	q13.3	MAB21L1	Highly expressed in brain, especially cerebellum		[92]
		DCAMKL1	Microtubule associated protein	Migration of neurons	[92]
		SMAD9	Signal transduction		[92]
15	q11.2	NDN	Interacts with NGF	Neuron differentiation	[94]
		SNRPN	Splicing factor, small nucleolar RNA, UBE3A antisense	Brain-specific RNA splicing, regulation of UBE3A?	[94]
		SNURF	SNRPN upstream reading frame		[94]
		UBE3A	E3 ubiquitin protein ligase	Protein degradation and Long Term Potentiation	[78, 94]
	q12	GABRB3	GABA receptor subunit	Synaptic transmission	[78, 94]
		GABRA5	GABA receptor subunit	Synaptic transmission	[78, 94]
		GABRG3	GABA receptor subunit	Synaptic transmission	[78, 94]
16	p13.3	TSC2	GTPase-activating protein for RAP1and RAB5	Inhibition of cell growth, brain development	[57]
17	q11.2	NF1	GTPase-activating protein for RAS	Long term potentiation	[57]
18	q12.1	MAPRE2	Microtubule binding protein	Neurite outgrowth	Paper I
	q12.2	BRUNOL4	RNA binding protein	Differentiation and maintenance of neurons	Paper I
		SLC39A6	Zinc transporter		Paper I
		ZNF397	Transcription factor		Paper I
		ZNF396	Transcription factor		Paper I
		ZNF271	Transcription factor		Paper I
		ZNF24	Transcription factor		Paper I
		STATIP1	Scaffolding protein JAK-STAT signalling	neuronal and glial cell proliferation, survival and differentiation	Paper I
		GALNT1	O-glycosylation enzyme	Neuronal differentiation and migration	Paper I
	q21.1	ZBTB7C	Transcription factor		Paper IV
	q21.33	BCL2	Inner mitochondrial membrane protein	Inhibition of apoptosis	[95]
21	q22.2	PCP4	Highly expressed in brain		[78]
		DSCAM	Cell adhesion molecule	Axon guidance	[78]
X	p11.23	FTSJ1	RNA methyl transferase	Regulation of translation	[96]
		HDAC6	Tubulins deacetylase	Clearing of misfolded proteins	[96]
		PQBP1	Polyglutamin and RNA binding protein	Inhibits basal transcription	[96]
		GRIPAP	Nucleotide exchange factor for Ras in brain	Synaptic localisation of AMPA receptors	[96]
		SYP	Synaptic vesicle protein	Short- and longterm synaptic plasticity	[96]
	p11.3	MAOA	Enzyme	Degrades catecholamines and serotonin	[97]
	p21.3	ARX	homeobox transcription factor	Axonal guidance and neuronal maintenance	[36]
	p22.2	GRPR	GRP receptor	Long term potentiation and regulation of fear reaponse	[98]
	p22.31	VCX3A	Deleted in mental retardation		[99]
	p22.31-22.32	NLGN4	Post synaptic membrane protein	Synaptogenesis	[34, 99]
	q13	NLGN3	Postsynaptic membrane protein	Synaptogenesis	[34]
	q27.3	FMR1	RNA-binding protein	Synaptogenesis and long term potentiation	[57]
	q28	MECP2	Methylated CpG island binding protein	Transcriptional silencer, synaptogenesis, neuroprotection	[54, 100]

Table 1 continued. Chromosome 9-22 and X. Chromosome position, protein function and inferred biological pathway for putative susceptibility genes for ASDs. Chromosome position refers to UCSC march 2006 (hg18). Papers I-IV refers to papers presented in this thesis.

2 OBJECTIVES OF PRESENT STUDY

Understanding the pathophysiological mechanisms underlying the development of ASDs will hopefully reveal information on the abnormal and normal development of the brain, which is not only of academic interest but might also reveal medical treatment strategies for ASDs and comorbid brain disorders. Moreover, having a child with an ASD can be a lifelong challenge and hence, identifying susceptibility genes for ASDs is important in making accurate genetic counselling and prenatal diagnosis possible for the involved families.

The aim of the present study was to identify putative susceptibility genes for ASDs by characterizing chromosomal rearrangements at the molecular level.

Four ASD patients, each with at least one chromosomal rearrangement, were included in our study. The general inclusion criteria were:

1. Apparent overlap in location of inferred putative susceptibility genes for ASDs and cytogenetically determined breakpoints.
2. Apparent overlap in location of previous linkage results for ASDs and cytogenetically determined breakpoints.
3. Apparently identical breakpoints in unrelated patients with similar phenotypes.

de novo rearrangements are preferably chosen for candidate susceptibility gene identification because the concomitant *de novo* nature of phenotype and genotype infers a direct connexion. Moreover, inherited rearrangements that segregate with a phenotype in a family are also widely used. In addition, for complex disorders like ASDs identifying individuals with inherited chromosomal rearrangements from both parents would agree with the suggested multigenic inheritance pattern.

All patients investigated in this study had apparently balanced chromosome aberrations. The patients and inclusion criteria were as follows:

Paper I : t(5;18)(q34;q12.2)*de novo*

In addition to the *de novo* nature of this translocation it was of interest in concern to ASDs since several neurotransmitter receptor genes are located at 5q34 and deletions of 18q12 had been reported to cause very mild dysmorphic features hardly disclosed at birth, psychomotor delay, hypotonia, ataxia, some degree of mental retardation and behavioural abnormalities [101].

Paper II: t(3;8)(q21;q24)*de novo*

This translocation was *de novo* in origin. Moreover, it was interesting in concern to ASDs because the chromosome 8 breakpoint apparently coincided with a suggestive linkage interval [102] and the breakpoint on chromosome 3 was seemingly shared by another ASD patient in the Mendelian Cytogenetics Network Database: (MCNdb, <http://www.mcndb.org>).

Paper III: t(4;16)(q27;p13.3)mat

Linkage studies for ASDs had identified both 4q26 and 16p13 as regions harbouring possible susceptibility genes [103, 104]. Accordingly, this translocation was of interest in concern to ASDs.

Paper IV + V: t(9;18)(p22;q21.1)pat, inv(10)(p11.2q21.2)mat

Linkage studies had suggested 9p22, 10p12, 10q22 and 18q21 as a candidate susceptibility region for ASDs, however not all with LOD scores above 1.5 [105-108]. Moreover, deletion of chromosome band 18q21 had been reported to cause mild to profound mental retardation [109]. In addition, the inheritance of both a paternal translocation as well as a maternal inversion as described in **paper IV** agrees with the most often suggested multigenic inheritance pattern for ASDs.

3 PAPER I

Identification of several putative susceptibility genes for childhood autism

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ABSTRACT

Autism spectrum disorders (ASDs) are a heterogeneous group of disorders with unknown aetiology. Even though ASDs are suggested to be amongst the most heritable complex disorders, only a few reproducible mutations leading to susceptibility for ASD have been identified. In a female patient with childhood autism we identified an apparently balanced de novo translocation, t(5;18)(q34;q12.2). Further analyses revealed a 3.2 Mb deletion at the 18q breakpoint and a 1.27 Mb deletion on chromosome 4q35, which was not involved in the translocation. One of the 17 genes within the deleted region at 18q was *BRUNOL4* (*bruno-like 4 RNA binding protein*), which is of particular interest with regards to ASD. *BRUNOL4* is highly expressed in the limbic system, cerebellum and cerebral cortex which are the areas most consistently found to be affected in neuropathological investigations of ASD brains. Furthermore, presence of a 4.4 Mb stable gene desert 5' to *BRUNOL4* with five ultra conserved sequences (UCSs) around *BRUNOL4* suggests that it is an important developmental gene. We sequenced the coding region of *BRUNOL4* and the five UCSs around the gene in 157 autistic patients, and identified three nucleotide changes in two UCSs that were not present in 167 controls analyzed. We suggest *BRUNOL4* as a susceptibility gene for at least some forms of ASDs.

KEYWORDS

Autism, *BRUNOL4*, ultra conserved sequences, chromosome 18, translocation, deletion.

INTRODUCTION

Childhood autism is a neurodevelopmental disorder with onset in early childhood. It is characterized by impairment of social interaction and communication accompanied by stereotypic behaviour or interests with onset of symptoms before the age of three. The prevalence of childhood autism is estimated to be between 10 and 60 in 10.000 [5-7] with a male to female ratio of four to one [2]. Cumulative evidence from family and twin studies suggests that childhood autism is amongst the most heritable complex disorders with a concordance rate of 60-90% in monozygotic twins and a recurrence rate of 2-3% in siblings of affected probands [1, 2]. The mode of inheritance is not known but the variation in phenotype reflects genetic heterogeneity [105]. The most parsimoniously suggested genetic model involves several epistatic genes but it is not known whether these genes are always the same or vary among

families [2]. Moreover, since the monozygotic concordance rate is not 100% it is likely that environmental factors also play a part in the development of autism in at least a subset of patients. Finally, some cases of autism may arise as a Mendelian or near-Mendelian disorder through for example chromosomal rearrangements that are seen in approximately 3% of patients with autism spectrum disorders (ASDs) [21, 48]. In this study we present a female childhood autism patient with a *de novo* translocation, t(5;18)(q34;q12) and two submicroscopic deletions, one at the 18q translocation breakpoint and the other in a chromosome not involved in the translocation. Among the genes deleted one of them, *BRUNOL4*, is of special interest in regards to autism.

METHODS

Patients

The translocation patient:

The patient is a 38 year old Danish woman with an apparently balanced *de novo* translocation t(5;18)(q34;q12). She is the first of two children of unrelated and healthy parents. Her younger sister is phenotypically normal. At birth, her mother was 21 and her father was 24 years old. She was born at term after a pregnancy with reduced intrauterine movement as described by the mother. Delivery was prolonged and asphyxia was noted at birth. Birth weight was 2500 g and birth length was 50 cm. Mild cerebral palsy, hyper flexible joints, excessive myopia (dioptre: -12, -11) as well as a hypersensitivity to sounds was later observed. She did not have any dysmorphic features. She sat at 9 months of age, walked alone at 17 months and said her first words and sentences at 42 months of age. At 3 years of age she was diagnosed with childhood autism. She attended a school for autistic children until the age of 18 where she moved to an institution for adults diagnosed with autism. At the age of 34 she was tested with an Autism Diagnostic Observation Schedule (ADOS) [110] module 4 for adults with fluent speech and her mother was interviewed with Autism Diagnostic Interview-Revised (ADI-R) [111]. Both tests clearly showed that the patient fulfilled the criteria for childhood autism diagnosis as defined in the International Classification of Diseases, tenth revision (ICD-10). In the ADOS test, the patient scored 7 points in both the “communication” area (autism cut-off 3) and “qualitative impairment in reciprocal social interaction” area (autism cut-off 6) and thus the total score was 14 points (autism cut-off 10). The results from the ADI-R gave equivalently a score of 27 in the “qualitative impairment in reciprocal social interaction” area (autism cut-off 10); a score of 18 in the “communication” area (autism cut-off 8); and a score of 9 in the “restricted, repetitive behaviour” area (autism cut-off 3). At the same time the

Wechsler Adult Intelligence Scale-Revised (WAIS-R) showed a verbal IQ of 78, a performance IQ of 105 and a full IQ of 88. Today, she lives in a small sheltered house for adult autistic patients.

The National Ethics Committees and the Danish Data Protection Agency approved the study, and informed consent was obtained.

DNA for sequencing analysis:

For mutation screening DNA from a total of 157 autistic patients was collected. One hundred autistic patients were recruited at the Hospital Pediátrico de Coimbra, originating from mainland Portugal and the Azorean islands. The male to female ratio was 4.8:1, and the ages ranged between 2-18 years (mean age 6.8 years). Idiopathic subjects were included after clinical assessment and screening for known medical and genetic conditions associated with autism, including testing for Fragile X mutations (FRAXA and FRAXE), chromosomal abnormalities, neurocutaneous syndromes, endocrine (thyroid function screening) and metabolic disorders. Another 35 children diagnosed with childhood autism were recruited at child psychiatric hospitals in the western part of Denmark (Jutland) (age range 3-30 years, with mean age of 10 years and male-female ratio of 3:1). Part of the sample has been described elsewhere [112]. 13 autistic patients were ascertained at the John F. Kennedy Institute, Glostrup, Denmark. These patients were all unrelated and were part of the IMGSAC group. Assessment methods and inclusion criteria has previously been described [108]. 11 of the 13 patients had siblings and some even additional relatives with a Pervasive Developmental Disorder (PDD) diagnosis. Four patients diagnosed with childhood autism were collected at Psychiatric Hospital, Frederiksborg Amt, Denmark. In all of these 152 patients, autism was diagnosed in accordance with DSM-IV or ICD-10 criteria using ADI-R in addition to ADOS or the Childhood Autism Rating Scale (CARS) [113, 114]. In addition, five DNA samples from ASD patients with chromosomal rearrangements were included. Two of these DNA samples were collected at The Wilhelm Johannsen Center for Functional Genome Research, University of Copenhagen, Denmark and were from Danish males diagnosed with childhood autism in accordance with ICD-10. Two DNA samples from a Swedish, male twin couple were collected by Ulf Kristoffersson at the Department of Clinical Genetics, University Hospital Lund, Sweden and one male DNA sample was collected by James Lespinasse at Laboratoire de Genetique Chromosomique, Centre hospitalier Chambéry, Chambéry, France. These three patients have an ASD diagnosis but have not been diagnosed according to ICD-10 or DSM-IV.

A total of 167 DNA samples from normal controls were collected. 96 DNA samples were collected at Statens Serum Institut, Copenhagen, Denmark. DNA was extracted and amplified from blood spots on filter-paper used in the Danish newborn screening program. The study was totally anonymous and the samples were taken randomly from a pool of daily routine samples. 71 normal DNA samples (32 females, 39 males) were collected at Department of Medical Genetics, Institute of Molecular and Cellular Medicine, University of Copenhagen, Denmark. These DNA samples were parent DNA from a larger collection of Danish non-consanguineous families with at least four children and no known diseases.

Whole Genome DNA amplification

The 100 DNA samples from Hospital Pediátrico de Coimbra and some of the 35 DNA samples from child psychiatric hospitals in the western part of Denmark were genome amplified by us using GenomiPhi™ DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK).

The kit Phi29 WGA (Amplicon, Brighton, UK) was used to amplify DNA from blood spots at Statens Serum Institut.

Fluorescence *in situ* hybridization (FISH)

Metaphase chromosomes were prepared from peripheral blood lymphocytes and the karyotype of the translocation patient was determined by G-banding. FISH was performed using bacterial artificial chromosome (BAC) clones from the RPCI-11 library and standard protocols. The BAC clones were obtained from the MCN reference centre at Max Planck Institute for Molecular Genetics, Berlin (<http://www.molgen.mpg.de/~cytogen/>) or the Wellcome Trust Sanger Institute, Cambridge (http://www.sanger.ac.uk/cgi-bin/software/archives/new_clone_login.cgi). 250 ng BAC DNA was biotin-14-dATP labelled by nick translation and hybridized to patient metaphase chromosomes. Signals were visualized using avidin-FITC detection system and chromosomes were counterstained with DAPI. Signals were investigated using a Leica DMRB epifluorescence microscope equipped with a Sensys 1400 CCD camera (photometrics) and an IPLab Spectrum imaging software (Vysis).

Microarray-based CGH

Array-based comparative genome hybridization (array-CGH) with a whole genome 32K BAC array was performed for the translocation patient. The method is described in [115].

Real-time quantitative PCR analysis (Q-PCR)

mRNA and total RNA panel (tissues used listed in supplementary table 1A+1B) (Clontech, CA, USA) was DNase treated before cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) was performed according to manufacturer's protocol. cDNA was checked for DNA contamination by PCR with three primer pairs located in a region with no known genes (supplementary table 5). Q-PCR analysis was carried out on a DNA Engine Opticon 2 (Bio-Rad, Göteborg, Sweden) using LightCycler FastStart DNA Master^{PLUS} SYBR GreenI (Roche, Hvidovre, Denmark). From 12 analyzed housekeeping genes, six were selected for normalization by using the BestKeeper software [116]. Primers used are listed in supplementary table 2.

In situ hybridization

12 µm thick coronal cryostat sections of the mouse brain were prepared and mounted on Superfrost Plus[®] slides. The sections were hybridized as previously described [117] with three 38-mer ³⁵S-labeled oligonucleotide probes complementary to *BRUNOL4* mRNA. An oligonucleotide probe was used for sense control (supplementary table 3).

The commercially synthesized probes were suspended in sterile DEPC-water to a concentration of 5 pmol/µl. Five µl of the probe was then labeled with [³⁵S]dATP with terminal transferase (Roche, Basel, Switzerland) to a specific activity of 1 x 10¹⁸ dpm/mol. Frozen sections were fixed after thawing for 5 min in 4% paraformaldehyde in PBS, washed twice in PBS, and acetylated for 10 min in 0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine. Sections were then dehydrated in ethanol, delipidated in chloroform, rehydrated partially and allowed to dry. Section hybridization was performed in a humid chamber overnight at 42°C with 200 µl labelled probe in 4X SCC buffer containing 50% (v/v) formamide, 1X Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 10% (w/v) dextran sulfate, 10 mM DDT, 0.5 mg/ml salmon sperm DNA and 0.5 mg/ml yeast tRNA. Slides were washed in 1X SCC at 55°C and then room temperature, and finally rinsed in distilled water. Sections were exposed to an X-ray film for 1 to 2 weeks. The X-ray film was developed by using a commercial developing machine.

Images of the sections on X-ray film were transferred to a computer using a light box, a COHU 4912 high performance CCD camera, and Image 1.42 software (Wayne Rasband, NIH, Bethesda, MD). The pictures were mounted in Adobe Photoshop 7.0.

Denaturing high performance liquid chromatography (dHPLC)

Mutation analysis of the ultra conserved sequences (UCS) around *BRUNOL4* included the sequences originally defined by Bejerano [118]. 157 patients within the ASD spectrum were screened for mutations together with 167 normal controls. Primers were designed using the Oligo6 software (Molecular Biology Insights, USA) and are available online in supplementary table 4. Melting temperature (T_m) was predicted based on the fragment sequence by using:

<http://insertion.stanford.edu/melt.html>. Sequences were analyzed using standard operating procedure of a Varian Helix™ DHPLC analysis system with a Helix™ DHPLC column (Varian Inc. CA., USA). Subsequent sequencing was performed as described below.

Sequencing

Mutation analysis of the *BRUNOL4* gene was done by direct sequencing in 157 ASD patients. We analyzed all coding exons and splice sites corresponding to clone NM_020180. Primers were designed using the Oligo6 software (Molecular Biology Insights, USA) and are available online in supplementary table 6. The sequencing reactions were carried out by Macrogen Inc in Korea (<http://www.macrogen.com/>) and ChromasPro version 1.33 (Technelysium Pty Ltd, Australia) was used to analyze the data.

RESULTS

FISH analyses

FISH was carried out to characterize the breakpoints of the translocation patient. On chromosome 5 the BAC clone RP11-541P9 (AC113414) was spanning the breakpoint, while RP11-256N5 (AC091921) was proximal and RP11-2A20 (AC091930) was distal. No known genes were located within this breakpoint region. On chromosome 18 a microdeletion of approximately 3.2 Mb containing 17 annotated RefSeq genes was identified (supplementary table 7). FISH on parent chromosomes

with BAC clone RP11-797E24 (AC090386) located within this deletion showed that the deletion was *de novo* in origin.

FISH with clone RP11-121i22 confirmed the presence of a deletion on chromosome 4q35 as identified by array-CGH and showed that the deletion was inherited from the father.

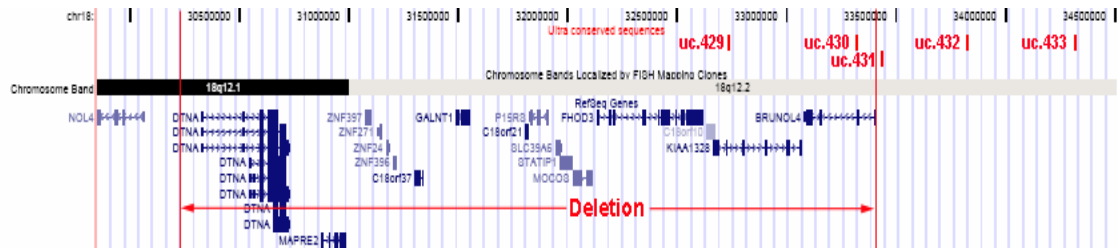


Figure 1. The 3.2 Mb deletion involving 17 genes on chromosome 18q12 in the translocation patient. RefSeq genes and ultra conserved sequences (uc.429 – uc.433) are shown (UCSC Genome Browser, May 2004 assembly). Ultra conserved sequences are numbered in accordance with [118].

Array-CGH

Array-based comparative Genome Hybridization (array-CGH) was performed for the translocation patient. In addition to the deletion on chromosome 18q12 ranging from RP11-667A14 to RP11-95G24 (chr18:30,09-33,35 Mb; NCBI35; HG17) a deletion of approximately 1.2 Mb was identified at 4q35 (RP11-215A19 to RP11-746B09; chr4:187,648-188,915 Mb, NCBI35; HG17). This deletion comprised two RefSeq genes: *MTNR1A* (Melatonin receptor 1A) and *FAT* (Human homolog of the *Drosophila fat* tumor suppressor gene).

Q-PCR

The information on most of the genes deleted at 18q12 was sparse. We therefore performed real-time quantitative PCR (Q-PCR) on 14 of the 17 genes to pinpoint which genes were possible ASD susceptibility genes based on their tissue expression profile. Dystrobrevin alpha (*DTNA*) and polypeptide N-acetylgalactosaminyltransferase (*GALNT1*) were already well described and were therefore not included and *KIAA1328* was not annotated at the time of our investigations. The expression pattern of the 14 genes in human brain tissue is shown in figure 2.

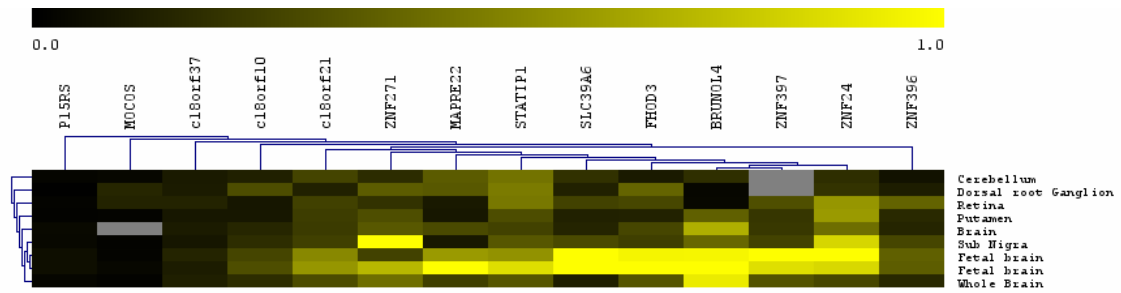


Figure 2. Cluster analysis of brain specific, normalized Q-PCR data for 14 of 17 genes within the deletion of our translocation patient. Yellow is high expression, black is low expression and grey refers to expression outside of the standard curve. Multiple Array Viewer has been used to produce this figure. The Q-PCR data is listed in supplementary table 1A + 1B.

In situ with mRNA from *Brunol4*

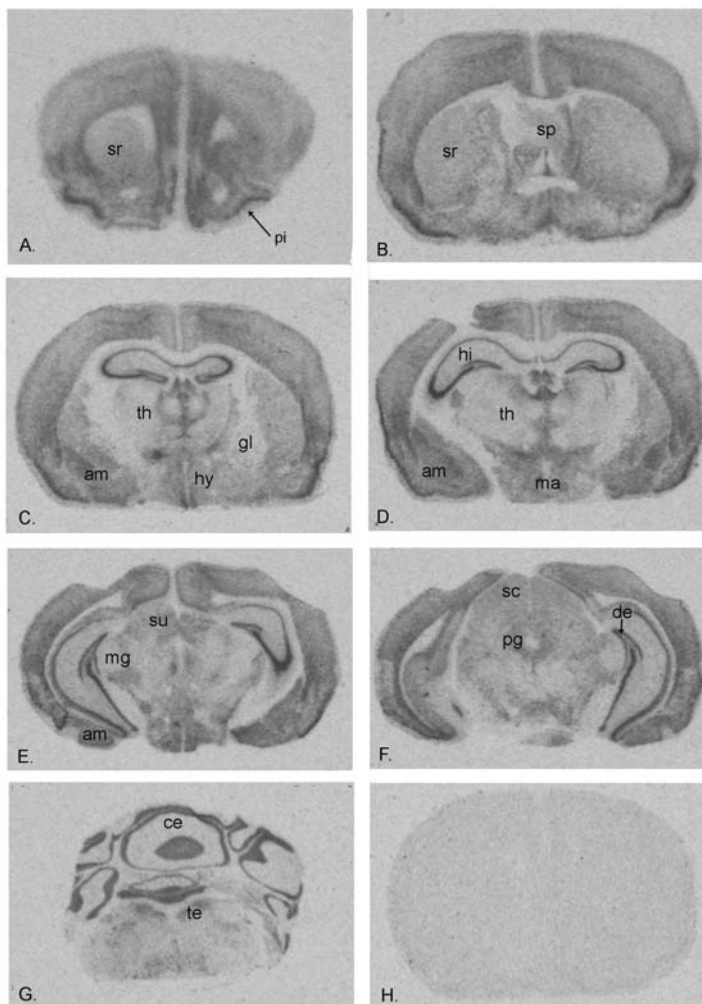


Figure 3. In situ hybridization for mRNA transcript of *Brunol4* on coronal sections of the mouse brain. The montage shows images on x-ray films of hybridized coronal sections from rostral to caudal levels (A-G). A coronal section of the forebrain, hybridized with a sense probe, is seen in H. am = amygdala; ce = cerebellum; de = dentate gyrus; hy = hypothalamus; hi = hippocampus; ma = mamillary nuclei; mg = medial geniculate body; pi = piriform cortex; sp = septum; sr = striatum; su = superior colliculus; te = tegmental area; th = thalamus.

A strong hybridization signal for Brunol4 was found in many areas of the mouse brain (figure 3): the neocortex, striatum, cerebellum, amygdala, hippocampus and piriform cortex. A strong hybridization signal was also seen in the hypothalamus including the mammillary body. Hybridization with a sense probe did not result in any signal (figure 3, H). These results agree with previous findings [119-121].

Mutation Screening of *BRUNOL4*

All the 12 coding exons and splice sites for *BRUNOL4* (NM_020180) were sequenced in 157 patients with ASDs. Three new silent nucleotide changes were identified within the coding region of *BRUNOL4*: ss67005831, ss67005837, ss67005840.

Mutation Screening of Ultra Conserved Sequences

Five ultra conserved sequences (UCS) have previously been identified around *BRUNOL4* (figure 1) [118]. Two of these UCSs were deleted in the translocation patient (uc.429 and uc.430). All five UCS's were screened for mutations in 157 ASD patients using dHPLC and samples with possible mutations were sequenced subsequently. Three nucleotide changes were identified in three unrelated autistic patients: a C>T nucleotide change was identified in uc.430 (ss67005811) and a C>A nucleotide change (ss67005820) as well as a T>C (ss67005817) nucleotide transition was identified in uc.432. These nucleotides are conserved in mouse, rat, rabbit, dog, armadillo, elephant, opossum, and chicken. The T>C substitution in uc.432 was inherited from the phenotypically normal father. The parental origin for the remaining changes is not known since DNA from the parents of these patients was not available. These sequence changes could not be identified in 167 controls. Moreover, a common nucleotide change was identified in uc.432 in both patients and controls. An additional nucleotide change (ss68074235) was identified in uc.430 in a control subject.

DISCUSSION

In a female patient diagnosed with childhood autism we identified a 1.27 Mb deletion on chromosome 4q35 encompassing *MTNR1A* and *FAT* and a 3.2 Mb deletion on chromosome 18q12.1-q12.2 encompassing 17 known genes (figure 1) in addition to the already identified *de novo* translocation t(5;18)(q34;q12). Since chromosomal imbalances are a known cause of mental retardation and other congenital anomalies [122] it is likely that deletion of one or more genes in this patient may lead to the observed autism phenotype due to haploinsufficiency.

On chromosome 4, two known genes were deleted: *MTNR1A* and *FAT*. In addition, approximately 900 kb of a gene desert located 5' to these genes is deleted. According to the Database of Genomic Variants [123] two deletions have previously been identified in this area: a normal control has a deletion overlapping the gene desert [124], whereas a patient with a chromosomal rearrangement and unknown phenotype has a deletion including *MTNR1A* and *FAT* in addition to six other genes [125]. Moreover, an additional 4q deletion possibly containing the *FAT* gene has been published in a patient with schizoaffective disorder [126] and Blair et al showed that *FAT* and its protein partners may be components of a molecular pathway involved in susceptibility to bipolar disorder [127]. Several lines of evidence suggest that some susceptibility genes for ASDs, schizophrenia and bipolar disorder are shared [10, 13, 20] intimating that haploinsufficiency of *FAT* might contribute to the phenotype of our patient.

A large number of deletions of varying size and location on the long arm of chromosome 18 have already been published [101, 128-134]. However, most case reports that describe deletions that apparently overlap with our deletion have not been fine mapped, and therefore offer a poor resolution of the deletion breakpoints [101, 131-133], which renders them difficult to use for genotype/phenotype correlations. However, the most common features of 18q12 deletion patients described in the literature are very mild dysmorphic features hardly disclosed at birth, psychomotor delay, hypotonia, ataxia, some degree of mental retardation and behavioural abnormalities [101]. These features indicate that one or more genes within this region are crucial for development and normal function of the brain. More recently, McEntagart and colleagues have reported a patient with del(18)(q11.2q12.2) which was overlapping with the deletion described in this paper (figure 4) [135]. The borders of the deletion described by McEntagart are uncertain (figure 4) but the deletion certainly fully overlaps the deletion described here. Thus, deletion of the 17 known genes identified in our translocation patient is common in both patients.

The phenotypes of our translocation patient and McEntagart's patient are very similar (table 1). Even though McEntagart's patient does not have an ASD diagnosis, he has delayed psychomotor and language development and some degree of behavioural difficulties which are core symptoms in ASDs. The overlap in genotype and phenotype of our translocation patient and McEntagart's patient makes it reasonable to conclude that haploinsufficiency of one or more genes within our deletion is most likely causing this shared phenotype.

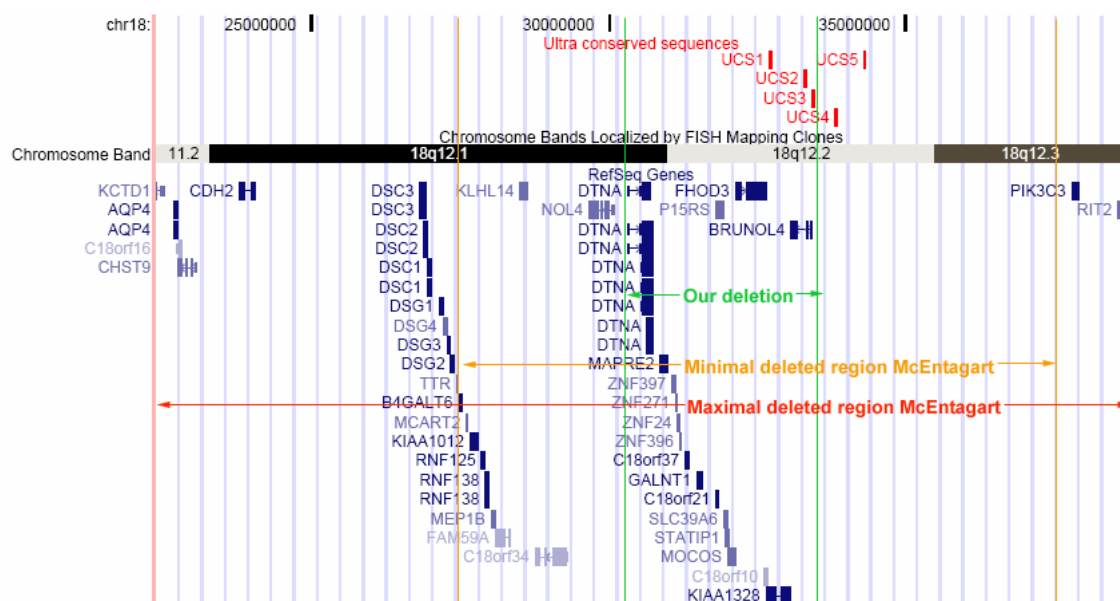


Figure 4. Screen plot from the UCSC genome browser (May 2004) showing the deletion in our translocation patient together with the minimal and maximal deletion from McEntagart's case report.

Phenotype	Our patient	McEntagart [135]
Hypotonia	No	Yes
Febrile convulsions	No	Yes
Cerebral palsy	Yes	No
Delayed psychomotor development	Yes	Yes
Delayed language development	Yes	Yes
Behavioural difficulties	Yes	Yes
IQ	85	61
Autism	Yes	
Myopia	Yes	

Table 1. Phenotype comparison of our translocation patient to McEntagart's patient.

Some of the 17 genes within the shared deletion can readily be excluded as possible susceptibility genes. The Database of Genomic Variants [136] states that a part of *c18orf10* and *KIAA1328* [137] is deleted in a normal control subject and that *FHOD3* is deleted in two normal control subjects in two separate studies [137, 138] suggesting that haploinsufficiency of these genes in our translocation patient and in McEntagart's patient is not causing the shared phenotype. Moreover, our Q-PCR data imply that *c18orf37*, *P15RS* and *MOCOS* are not susceptibility genes since they are not expressed in the brain (figure 2). Our Q-PCR data also show that several genes are expressed in the brain and thus may contribute to the phenotype (figure 2). When considering the biological processes of the proteins encoded by these genes they might all contribute to the phenotype: a zinc transporter that assures cofactors for

hundreds of cellular enzymes (*SLC39A6*) [139]; four zinc finger transcription factors (*ZNF397*, *ZNF396*, *ZNF271*, *ZNF24*); a scaffolding protein of the JAK-STAT signalling pathway suggested to be involved in neuronal and glial cell proliferation, survival and differentiation (*STATIP1*) [140-142]; an O-glycosylating enzyme that might enable cells to adhere, differentiate and migrate (*GALNT1*) [143] and a microtubule associated protein that is possibly involved in the development of neuronal processes (*MAPRE2*) [144]. However, especially *BRUNOL4* is interesting with respect to the shared phenotype. The expression pattern of *BRUNOL4* (supplementary Q_PCR data, figure 3, and [119]) mirrors the brain areas most consistently found to be affected in neuropathological investigations of autism, namely: the limbic system, cerebellum and cerebral cortex [42].

BRUNOL4 belongs to the bruno-like elav (embryonic lethal abnormal visual system) family of genes [119, 145]. This gene family encodes RNA binding proteins containing three highly conserved RNA recognition motifs that are important in posttranscriptional regulation of gene expression, such as alternative mRNA splicing, regulation of translation and rate of mRNA turnover [119, 145]. Similar gene functions have been reported for genes affected in X-linked Mental Retardation (XLMR): *FMR1*, *PQBP1*, *FTSJ1* [146]. This is not surprising since mutations in some genes (*ARX*, *NLGN4*, *MECP2*) [35, 36, 147] have been reported to cause both ASDs and mental retardation, suggesting an overlap in aetiology of these phenotypes. Spontaneous mutations in vertebrate elav-like genes have according to Antic et al [145] previously not been identified. However, experiments with deletions of the entire *elav* gene in *Drosophila* strongly suggest a role for *elav* in differentiation and maintenance of neurons in the CNS, as well as for the embryonic development of the eye [148-150]. The phenotype of our patient suggests a similar role for *BRUNOL4* in humans even though haploinsufficiency for one or more of the additional genes within the deletion may also play a part as described above.

Several lines of evidence support the conclusion that *BRUNOL4* is an important developmental gene. There is a 4.4 Mb large evolutionary stable gene desert located at the 5'-end of the gene [151]. Stable gene deserts (in contrast to variable gene deserts) are defined as having a density of more than 2% evolutionary conserved regions (>100 bp and >70% identity in a sliding window) when comparing the genome of chicken and human [151]. Stable gene deserts presumably contain long distance transcriptional regulatory elements since some stable gene deserts include regions that have previously been shown to harbour such elements [151]. Moreover, comparative sequence analysis of human gene deserts with homologous

Fugu counterparts revealed that 98% of the total number of evolutionary conserved regions identified in gene deserts was located in stable gene deserts, and three times as many conserved elements within stable gene deserts compared to variable gene deserts have regulatory potential [151]. In addition, only 2 out of 172 stable gene deserts are interrupted by a synteny breakpoint, suggesting that stable gene deserts are functionally linked to at least one of the flanking genes [151]. Stable gene deserts are primarily positioned next to genes involved in transcriptional regulation, DNA binding, regulation of metabolism and development [151]. In addition, five ultra conserved non-coding sequences (UCS) are located close to *BRUNOL4* within a region spanning from 343 kb downstream within intron 5 of KIAA1328 to 918 kb upstream within the gene desert (figure 1). The UCSs are defined as sequences ≥ 200 bp with 100% identity in the human, mouse and rat genome [118, 152]. This makes UCSs more highly conserved between species than proteins and thus suggests a very important function in the human genome [118, 152]. Some UCSs have been shown to possess enhancer activity [153], suggesting that UCSs are involved in regulation of gene expression. UCSs appear to be associated with genes involved in RNA processing or in regulation of transcription and development [118, 152]. Thus, the presence of five UCSs around *BRUNOL4* substantiates the developmental importance of this gene.

The three nucleotide changes identified within the coding region of *BRUNOL4* are all silent and are therefore not likely to be involved in the pathogenesis of ASDs. However, the identification of three mutated nucleotides in two ultra conserved sequences can not readily be rejected as implicated in the ASD development since they were not identified in 167 control subjects. It is currently not known whether nucleotide changes in UCSs can contribute to disease development. Richler and colleagues conducted a similar analysis of UCSs on chromosome 7q and found several nucleotide changes both in autism patients and controls [154]. They conclude that their findings are probably of no relevance to the development of autism because the findings are rare and some changes are even inherited from phenotypically normal parents. Likewise, our results show that mutations in the coding region of *BRUNOL4* and the five surrounding UCSs are not frequent causes of ASDs. However, since the UCSs may be important in gene regulation and ASDs are considered to be both multifactorial and polygenic in aetiology it is possible that the identified mutations in the UCSs in conjunction with other genetic variations result in susceptibility to ASDs. Future functional assays with UCSs will hopefully shed light on

the function of these elements and thereby help to understand their possible role in the development of ASDs.

We have identified multiple possible susceptibility genes for ASDs within the deletion of our patient. This is in line with the most frequently suggested genetic model of autism as a complex, polygenic disorder. More experiments must be carried out before the importance of the identified genes in the aetiology of ASDs can be assessed.

ACKNOWLEDGEMENTS

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SUPPLEMENTS

Total RNA	BRUNOL4	c18orf10	P15RS	FHOD3	MAPRE2	ZNF271	ZNF397	c18orf37	STATIP1	MOCOS	SLC39A6	c18orf21	ZNF24	ZNF396
Fetal brain	3467.06	23.83	27.46	133.14	46.65	4.45	7.08	4.56	7.75	1.61	164.09	22.46	151.34	31.84
Retina	136.89	7.88	9.45	39.07	7.92	3.37	2.24	4.39	6.38	10.42	43.66	10.82	89.93	32.85
Brain	2464.01	13.35	18.72	38.69	22.59	5.88	1.59	3.37	3.56	NA	23.87	9.93	66.47	12.53
Putamen	1358.19	8.26	7.97	19.12	7.18	5.25	1.53	3	4.11	1.47	22.37	10.26	92.08	13.95
Sub Nigra	1456.02	15.66	16.49	34.94	7.64	16.74	1.9	2.86	4.64	1.4	48.72	10.71	127.71	23.3
Dorsal root Ganglion	112.62	26.47	4.49	54.96	26.62	6.11	NA	3.37	6.55	11.35	22.44	5.68	31.44	9.9
Fetal brain	3567.89	26.7	27.14	137.83	76.26	12.14	6.23	3.49	11.72	2.48	167.45	24.72	129.27	30.88
Cerebellum	671.68	11.44	10.9	14.66	27.81	2.98	NA	3.35	6.09	2.36	32.19	11.35	28.25	6.27
Whole Brain	3306.18	15.81	12.73	46.03	19.33	7.31	2.34	3.89	4.54	1.28	20.57	12.7	43.18	13.59
Fetal liver	NA	7.33	3.01	1.56	3.62	2.68	NA	2.61	3.05	37.44	3.35	3.37	6.9	1.06
Heart	2.95	1.73	1.36	54.18	4.67	NA	NA	1.52	NA	2.16	NA	NA	3.19	NA
Lung	NA	9.16	7.88	4.25	8.44	4.83	NA	4.12	5.53	14.5	14.61	9.26	76.17	22.06
Placenta	1	7.09	4.61	2.88	4.78	3.04	NA	8.92	3.77	72.88	24.87	5.16	30.91	2.6
Prostate	3.46	5.52	12.22	31.44	3.74	6.59	3.36	2.17	8.73	25.92	106.93	8.73	83.28	31.35
Salivary gland	2.03	4.12	3.93	9.53	1.04	1.52	NA	2.6	3.33	7.77	9.77	5.32	17.35	7.01
Skeletal muscle	NA	NA	3.69	40.51	1.38	5.68	NA	3.3	2.89	2.24	2.18	7.42	24.96	1.61
Spleen	NA	4.37	2.76	1.34	4.3	3.78	NA	4.21	7.44	1.13	6.27	8.58	64.38	5.13
Thymus	NA	12.06	2.13	4.67	7.9	2.76	NA	3.3	5.53	2.09	3.18	7.2	15.37	4.72
Trachea	2.44	6.84	1.78	12.37	1.49	2.11	NA	2.38	2.97	18.63	6.89	3.99	21.29	8.3
Uterus	2.44	6.68	2.55	6.44	2.61	3.49	NA	1.84	5.96	7.01	8.57	4.65	17.36	2.14
Colon	1.48	3.3	NA	3.23	NA	1.19	NA	NA	1.41	12.14	1.25	2.18	6.1	2.26
Small Intestine	4.96	3.65	3.04	4.36	1.73	1.76	NA	1.47	1.82	47.8	3.6	3.35	12.81	1.93
Stomach	2.16	4.81	4.36	5.62	2.23	2.96	NA	2.52	3.64	18.98	7.47	5.94	34.17	11.03
Pancreas	NA	4.66	5.29	NA	2.22	2.38	NA	1.33	4.95	9.56	15.81	9.78	30.38	13.96
Kidney	NA	4.09	3.07	36.75	2.41	2.59	NA	2.1	3.2	7.06	9.9	7.22	24.34	19.64
Spinal cord	141.99	13.16	452.38	21.33	4.4	4.52	NA	1.81	4.38	2.41	21.22	5.45	55.12	8.84
Testis	118.89	86.05	23.98	15.88	2.73	5.25	1.47	30.66	13.37	19.6	28.73	41.72	63.87	83.52
Fetal liver	NA	6.79	1.78	1.3	3.85	2.4	NA	2.39	3.9	35.01	2.88	3.5	7.89	2.24

Supplementary table 1A. Q-PCR data from 14 genes within the deletion on 18q12.

Total RNA	BRUNOL4	c18orf10	P15RS	FHOD3	MAPRE2	ZNF271	ZNF397	c18orf37	STATIP1	MOCOS	SLC39A6	c18orf21	ZNF24	ZNF396
Fetal brain	0.972	0.277	0.061	0.966	0.612	0.266	1.000	0.149	0.580	0.022	1.000	0.538	1.000	0.381
Retina	0.038	0.092	0.021	0.283	0.104	0.201	0.316	0.143	0.477	0.143	0.266	0.259	0.594	0.393
Brain	0.691	0.155	0.041	0.281	0.296	0.351	0.225	0.110	0.266	NA	0.145	0.238	0.439	0.150
Putamen	0.381	0.096	0.018	0.139	0.094	0.314	0.216	0.098	0.307	0.020	0.136	0.246	0.608	0.167
Sub Nigra	0.408	0.182	0.036	0.254	0.100	1.000	0.268	0.093	0.347	0.019	0.297	0.257	0.844	0.279
Dorsal root Ganglion	0.032	0.308	0.010	0.399	0.349	0.365	NA	0.110	0.490	0.156	0.137	0.136	0.208	0.119
Fetal brain	1.000	0.310	0.060	1.000	1.000	0.725	0.880	0.114	0.877	0.034	1.020	0.593	0.854	0.370
Cerebellum	0.188	0.133	0.024	0.106	0.365	0.178	NA	0.109	0.455	0.032	0.196	0.272	0.187	0.075
Whole Brain	0.927	0.184	0.028	0.334	0.253	0.437	0.331	0.127	0.340	0.018	0.125	0.304	0.285	0.163
Fetal liver	NA	0.085	0.007	0.011	0.047	0.160	NA	0.085	0.228	0.514	0.020	0.081	0.046	0.013
Heart	0.001	0.020	0.003	0.393	0.061	NA	NA	0.050	NA	0.030	NA	NA	0.021	NA
Lung	NA	0.106	0.017	0.031	0.111	0.289	NA	0.134	0.414	0.199	0.089	0.222	0.503	0.264
Placenta	0.000	0.082	0.010	0.021	0.063	0.182	NA	0.291	0.282	1.000	0.152	0.124	0.204	0.031
Prostate	0.001	0.064	0.027	0.228	0.049	0.394	0.475	0.071	0.653	0.356	0.652	0.209	0.550	0.375
Salivary gland	0.001	0.048	0.009	0.069	0.014	0.091	NA	0.085	0.249	0.107	0.060	0.128	0.115	0.084
Skeletal muscle	NA	NA	0.008	0.294	0.018	0.339	NA	0.108	0.216	0.031	0.013	0.178	0.165	0.019
Spleen	NA	0.051	0.006	0.010	0.056	0.226	NA	0.137	0.556	0.016	0.038	0.206	0.425	0.061
Thymus	NA	0.140	0.005	0.034	0.104	0.165	NA	0.108	0.414	0.029	0.019	0.173	0.102	0.057
Trachea	0.001	0.079	0.004	0.090	0.020	0.126	NA	0.078	0.222	0.256	0.042	0.096	0.141	0.099
Uterus	0.001	0.078	0.006	0.047	0.034	0.208	NA	0.060	0.446	0.096	0.052	0.111	0.115	0.026
Colon	0.000	0.038	NA	0.023	NA	0.071	NA	NA	0.105	0.167	0.008	0.052	0.040	0.027
Small Intestine	0.001	0.042	0.007	0.032	0.023	0.105	NA	0.048	0.136	0.656	0.022	0.080	0.085	0.023
Stomach	0.001	0.056	0.010	0.041	0.029	0.177	NA	0.082	0.272	0.260	0.046	0.142	0.226	0.132
Pancreas	NA	0.054	0.012	NA	0.029	0.142	NA	0.043	0.370	0.131	0.096	0.234	0.201	0.167
Kidney	NA	0.048	0.007	0.267	0.032	0.155	NA	0.068	0.239	0.097	0.060	0.173	0.161	0.235
Spinal cord	0.040	0.153	1.000	0.155	0.058	0.270	NA	0.059	0.328	0.033	0.129	0.131	0.364	0.106
Testis	0.033	1.000	0.053	0.115	0.036	0.314	0.208	1.000	1.000	0.269	0.175	1.000	0.422	1.000
Fetal liver	NA	0.079	0.004	0.009	0.050	0.143	NA	0.078	0.292	0.480	0.018	0.084	0.052	0.027

Supplementary table 1B. Normalized Q-PCR data from 14 genes within the deletion on 18q12. Each gene is normalized to the highest expression for that gene

Gene name	Primers	Length
MAPRE2	Forward: 5' GCG AGA ACA ACA ACG ACA 3'	164 bp
	Reverse: 5' TCT TGG GTT ATC GAG GTA GAA T 3'	
ZNF397	Forward: 5' AGA GCA TCC CAA GAG TCA AC 3'	233 bp
	Reverse: 5' AGT TTC TCC CCT GTA GCA CTT 3'	
ZNF271	Forward: 5' AAT TAT GAA TCT CAG GAA CAC CAC 3'	237 bp
	Reverse: 5' TTG CCC ATA TTC GTC ACA G 3'	
ZNF24	Forward: 5' AGT TTG TTG CCA TCC TAC CC 3'	158 bp
	Reverse: 5' TAG TAC TTC CCG TTT TCG TCG 3'	
ZNF396	Forward: 5' TTG GGA AAG TCA TCA TCA CTC CTA ACA C 3'	492 bp
	Reverse: 5' CAT GAG CTG GCT ACT TGG CAA TTC 3'	
c18orf37	Forward: 5' ACC TGA AAC AAA TCC TCG CTT CT 3'	150 bp
	Reverse: 5' TCT GGG GGT CTG TGT AGT TGG 3'	
c18orf21	Forward: 5' CGA GAA GCG AGA AAC TAT ACA CTC 3'	105 bp
	Reverse: TCT GTT GCA TGT TTT ACA AGT GAT 3'	
P15RS	Forward: 5' CAT TCA CCA CCG TAA ACA C 3'	254 bp
	Reverse: 5' ACC TTT CTT CCC AAA TAG ATA AC 3'	
SLC39A6	Forward: 5' CAA CTA TCT CTG TCC AGC CAT CAT CAA CC 3'	259 bp
	Reverse: 5' AGC ATC ACC ACT CAA AGT CCC AAC G 3'	
STATIP1	Forward: 5' CCT TTC AGC CCT CCA TAC TTA C 3'	256 bp
	Reverse: 5' GCC ATC TGC GTG ACT GTC 3'	
MOCOS	Forward: 5' GTG GTG CGG ATT TAC AGC GAT T 3'	369 bp
	Reverse: 5' TGA TGA GTG CAG GCG AGT GTC 3'	
FHOD3	Forward: 5' GGA ACT GAT GAC TCG CCC AAT GT 3'	171 bp
	Reverse: 5' TGG GGT CAG GCC GCT CTT 3'	
c18orf10	Forward: 5' CTG GCA GAC CTG GAG GAC GAT A 3'	244 bp
	Reverse: 5' GGG TGA TGA GCA GGC GGT AA 3'	
BRUNOL4	Forward: 5' TTC CTC CCT TTC GGC TTC GTG 3'	193 bp
	Reverse: 5' ATC CTG CCC TGT GCG AGT CCT 3'	

Supplementary table 2. Primers used for Q-PCR are listed together with the size of the PCR product. A melting curve was made from 55°C-98°C.

Probe name	Sequence
Brunol4_antisense 1	5' TGG CGC AGG CAG CTA CTA CCT CCT CGG CTC TCG CTG TC 3'
Brunol4_antisense 2	5' GGC GGC GCA CGT CGT CCT CAG ATT GTT GCT TGT TGA GC 3'
Brunol4_antisense 3	5' CAG CTT GAT GGC ATC GTG GTC CTT CAT GGG AAT GGT CG 3'
Brunol4_sense 1	5' GAC AGC GAG AGC CGA GGA GGT AGT AGC TGC CTG CGC CA 3'

Supplementary table 3. Probes used for radioactive in situ hybridization of Brunol4.

Sequence name	Primer sequences	Length	Melting temp.
Uc429	Forward: 5' GCA CTG CTG GTG TTT AAG 3'	412 bp	57°C
	Reverse: 5' GGC TCT GTA TCT AAA AGT GTG 3'		
Uc430	Forward: 5' CCG TCC CAA CAT ACA CTC AC 3'	519 bp	57/60 °C
	Reverse: 5' AGA GGG GGA GGT TTA GGA TTT 3'		
Uc431	Forward: 5' TGT CAT AAT CAA AAG GCG GGA CTA CAG 3'	480 bp	57°C
	Reverse: 5' GCT TGC CCA CCA ACT AAC GAG AAC 3'		
Uc432	Forward: 5' GTC TAT CTT CTC ATG TGT CAG CAA CAA GT 3'	529 bp	57°C
	Reverse: 5' ACT CCT TCC ATT ACC CTC GCT AAA C 3'		
Uc433	Forward: 5' GCA AGC AAT TAC TGG CGA TTT GT 3'	424 bp	57°C
	Reverse: 5' TGT ATG GTG GCT CGC AAA GG 3'		

Supplementary table 4. Primers for dHPLC screening and sequencing of UCSs.

Sequence name	Primer sequences	Length
CHR7	Forward: 5' ATA AAG GTT CCT GTG ATG TCA G 3'	266 bp
	Reverse: 5' GTAAGTTATTTAGATGATTTTGAATGC 3'	
CHR13	Forward: 5' ATT AAA CAT GAT AAA AGG GAT TCT TAC 3'	213 bp
	Reverse: 5' ATA ATG AGC ACT TAG AGC AGG A 3'	
CHR20	Forward: 5' AAA CGT GTG CTC TTT TCC CCA TCT TAT 3'	354 bp
	Reverse: 5' GCT AGC CCT CTC TGG ATT CCT TCT TC 3'	

Supplementary table 5. Primers used for PCR check of genomic contamination of cDNA

Exon	Primers	Length
1	Forward: 5' TGT GTG TAT GCG TGT GCG TGT GT 3'	476 bp
	Reverse: 5' GCT TAG TCC GCC GCT CGT CA 3'	
2	Forward: 5' TGA CCC TTC TTG GCG CTT GCT 3'	339 bp
	Reverse: 5' GCA GGA ACT TCA CGG CCA CG 3'	
3	Forward: 5' GCA GCA TGT TGT GGG AGC GCG TGT 3'	354 bp
	Reverse: 5' CTC TGT CCC CAA CCC TCA GCC ACG AGA T 3'	
4	Forward: 5' CTC CCT TTC CCT GCC GCT CTC 3'	379 bp
	Reverse: 5' CAG GTG AAC GCA GAC GGG TGA 3'	
5	Forward: 5' CTC AAT CTC TGC CTC ACC CGT CT 3'	380 bp
	Reverse: 5' GGC ATT TTC CAC CTG GGT AAC CT 3'	
6	Forward: 5' GGA AGC CCA GGG ATG TTA AGG A 3'	464 bp
	Reverse: 5' GAG GGA GAG GGC AAG GGA TAG AG 3'	
7	Forward: 5' AGG GCT TTC TGG AGT GGT AGT TG 3'	368 bp
	Reverse: 5' CCG CTG GTA GCT GAA ATT AGG TC 3'	
8	Forward: 5' GGT CCA AGT CTA TCC GCA GGT 3'	316 bp
	Reverse: 5' AAA GAA TGT GCT GCA TAC GGA AAT 3'	
9	Forward: 5' ACT CAG GGT TTC TGT CTC GGA T 3'	283 bp
	Reverse: 5' GGT GTC ACA GGC GTG GAG 3'	
10	Forward: 5' CTT TTT GGC TGA TCT GCT TTT ACT GC 3'	387 bp
	Reverse: 5' CAC AAG AAG CGG ACC TCT ACC CTT A 3'	
11	Forward: 5' TCC CTG CTG CGC TGC CTA ACT 3'	235 bp
	Reverse: 5' TTA CTT TGG TTA GTC GCC CGA TCC AC 3'	
12	Forward: 5' AAA CCC AGG CCC TCG TCC CTC GTG TCT C 3'	350 bp
	Reverse: 5' AGG AGC AGG GCG AGG AGC AGG TTG AGC 3'	

Supplementary table 6. Primers for sequence analysis of *BRUNOL4*.

Probe Name	Accession nr.	Chromosomal position	Result
RP11-252a7	AC087814	29,545,452-29,727,556	Proximal
RP11-756m1	AC018972	29,664,156-29,824,757	Proximal
RP11-704i20	AP001131	29,718,544-29,869,997	Proximal
RP11-594p16	AC087397	29,764,080-29,949,991	Proximal
RP11-379l18	AC104985	29,917,692-30,089,878	Proximal
RP11-732e23	AC104988	30,047,928-30,219,417	Proximal
RP11-812d8	AP001125	30,184,237-30,378,471	Proximal
RP11-108g18	AC103768	30,197,999-30,378,471	Deleted
RP11-502p14	AC069131	30,262,228-30,440,155	Deleted
RP11-734h19	AP002895	30,373,720-30,529,001	Deleted
RP11-138h11	AC022601	30,478,145-30,636,743	Deleted
RP11-849j9	AP001165	30,834,503-31,010,999	Deleted
RP11-158h5	AC011815	30,973,085-31,129,339	
RP11-140n10	AC127506	31,089,012-31,240,845	
RP11-322e11	AC007998	31,171,484-31,379,284	Deleted
RP11-616h5	AC036180	31,241,591-31,424,228	
RP11-723j4	AP001905	31,773,519-31,995,543	Deleted
RP11-594b10	AP001155	31,995,859-32,163,467	Deleted
RP11-843p6	AP001401	32,443,591-32,645,221	
RP11-95o2	AC015961	32,925,213-33,089,023	Deleted
RP11-843p6	AP001401	32,443,591-32,645,221	Deleted
RP11-139k2	AQ383018/AQ383019	33,042,275-33,225,650	Deleted
RP11-797e24	AP001454	33,055,287-33,240,851	Deleted
RP11-680k13	AP002501	33,141,414-33,329,595	Deleted
G248P8789E10	G248P8789FE10/G248P8789RE10	33,308,719-33,352,196	Deleted
G248P85590D6	G248P85590RD6/G248P85590FD6	33,355,485-33,392,002	Deleted
RP11-1147p1	AC129908	33,276,774-33,467,582	Distal
RP11-298g9	AQ506634/AQ506631	33,276,776-33,467,576	Distal
RP11-661b20	AQ611604/AQ518661	33,417,988-33,578,547	Distal
RP11-776m20	AP001356	33,492,805-33,670,124	Distal
RP11-862c6	AP001912	34,142,324-34,340,289	Distal

Supplementary table 7. FISH results for delineation of deletion on 18q12.

4 PAPER II

***KCNQ3* is disrupted in a male patient with childhood autism**

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ABSTRACT

Autism spectrum disorders (ASDs) are a heterogeneous group of disorders with unknown aetiology. Even though ASDs are suggested to be amongst the most heritable complex disorders, only a few reproducible mutations leading to susceptibility for ASD have been identified. In a patient with childhood autism we have identified a *de novo* t(3;8)(q21;q24) translocation, where the 8q24 breakpoint disrupts the *KCNQ3* (*Potassium Channel, voltage-gated KQT-like subfamily, member 3*) gene. Since *KCNQ3* is a subunit of the M-channels that apparently are important for neural network excitability, postnatal brain development and cognitive performance, we suggest that *KCNQ3* may be a candidate susceptibility gene for ASD.

KEYWORDS

Autism, chromosome 8, *KCNQ3*, translocation.

INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders with early childhood onset and a lifelong persistence. They are characterized by impairments in reciprocal social interaction and communication as well as by stereotypic behaviour or interests. The prevalence of ASDs is estimated to be between 1 - 6 in 1000 [6] with a male to female ratio of 4:1 [2]. The genetic basis of ASDs plays a greater role in disease development than in any other common neuropsychiatric disorder since the concordance rate in monozygotic twins is 60-90% compared to a concordance rate of approximately 5% in dizygotic twins [1, 2]. The most consistently suggested genetic model of inheritance in ASDs involves numerous epistatic genes [2]. Whether these genes are always the same or vary between families is not known but it is likely that the variation observed in ASD phenotype reflects a correspondingly heterogeneous genetic basis. Despite the apparent robust genetic basis of ASDs no susceptibility genes or pathophysiological mechanisms have been unambiguously elucidated. We identified, and characterized, a *de novo* t(3;8)(q21;q24) translocation in a patient with childhood autism where the chromosome 8 breakpoint apparently coincided with a suggestive linkage interval identified in an a previous autism study [102].

METHODS

Patient

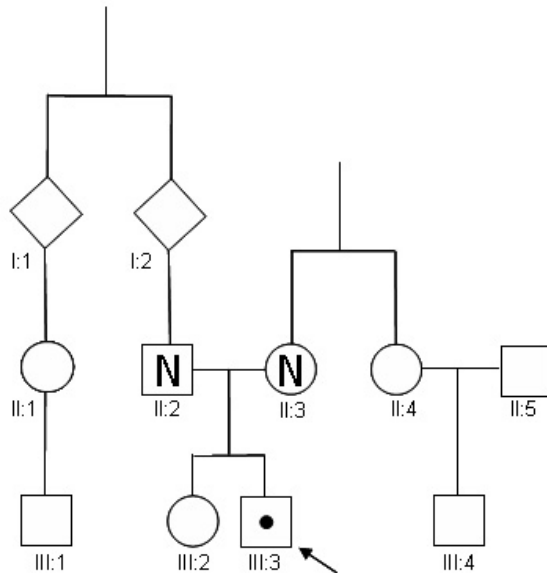


Figure 1. Pedigree showing translocation carriers with a dot and examined non-carriers with an N. Unmarked individuals have not been cytogenetically examined. III:1 has Asperger's syndrome, III:3 is the proband and III:4 has behavioural difficulties.

An eight year old Danish boy was diagnosed with childhood autism before the age of three. He is the second child of healthy, unrelated parents who both have academic degrees. The mother has seasonal affective disorder, which requires light therapy. He has a phenotypically normal older sister. The mother's sister's son (figure1, III:4) has behavioural difficulties and a paternal cousin has a son (figure 1, III:1) diagnosed with Asperger's syndrome. The pregnancy and delivery at gestational week 40+1 was uncomplicated. His weight at birth was 3900 g and his birth length was 53 cm. Apgar scores were 9/1 and 10/5. A weak quivering noted at birth and was present the first five weeks but was not observed at three months of age. No underlying physical abnormalities that could explain the quivering were identified. No dysmorphic features or other physical abnormalities have been identified in the patient. His development within the first year was normal. At approximately one year of age the parents noticed that he became withdrawn and it was difficult to obtain eye contact. A WISC-III (Wechsler Intelligence Scale for Children) test scoring performance on a scale between 1 and 19 (19 is the best and 10 is average for age) was carried out when the proband was eight years old. The results for language were: information 19, similarities 19, arithmetic 12,

vocabulary 13, comprehension 10, digit span 14, which adds up to a verbal IQ of 103. The results of performance were: picture completion 14, coding 3, picture arrangement 10, block design 11, object assembly 9, symbol search 8, mazes 16, which adds up to a performance IQ of 60. His full IQ was 79. Accordingly, the patient has a very uneven WISC profile with specific impairments of visuospatial skills. His motor development is normal. He is currently attending a kindergarten for normal children under supervision of a speech therapist. He is now able to speak in whole sentences but he only does so when it is necessary for fulfilling his needs. Cytogenetical analyses revealed a *de novo* t(3;8)(q21;q24) translocation and at the same time fragile X was excluded as causative of the identified autism phenotype. The parents and the older sister had normal karyotypes.

The National Ethics Committees and the Danish Data Protection Agency approved the study, and informed consent was obtained.

Fluorescence in situ hybridization (FISH)

FISH was performed using bacterial artificial chromosome (BAC) clones from the RPCI-11 library and standard protocols. The BAC clones were obtained from the MCN reference centre at Max Planck Institute for Molecular Genetics, Berlin (<http://www.molgen.mpg.de/~cytogen/>) or the Wellcome Trust Sanger Institute, Cambridge (http://www.sanger.ac.uk/cgi-bin/software/archives/new_clone_login.cgi). 250 ng BAC DNA was biotin-14-dATP labelled by nick translation and hybridized to patient metaphase chromosomes. Signals were visualized using avidin-FITC detection system and chromosomes were counterstained with DAPI. Signals were investigated using a Leica DMRB epifluorescence microscope equipped with a Sensys 1400 CCD camera (photometrics) and an IPLab Spectrum imaging software (Vysis).

Microarray-based CGH

Array CGH with a whole genome 32K BAC array was carried out as described previously [155].

RESULTS AND DISCUSSION

In a patient with childhood autism we identified an apparently balanced *de novo* translocation t(3;8)(q21;q24). The translocation was investigated further since the

chromosome 8 breakpoint apparently coincided with a suggestive linkage interval [102] and the breakpoint on chromosome 3 was seemingly shared by another autism patient in the Mendelian Cytogenetics Network Database

(MCNdb, <http://www.mcndb.org>). FISH was carried out to characterize both breakpoints. On chromosome 3 BAC clone RP11-332M2 was proximal, RP11-77P16 was distal and RP11-93K22, RP11-134C13, RP11-24P17, RP11-60C5 and Fosmid clone G248P83642D1 were spanning the breakpoint. No genes are located within this breakpoint region. On chromosome 8 BAC clone RP11-71N3 was proximal, RP11-454L8 was distal and clones RP11-721F1, RP11-960B24, RP11-668I20 and RP11-213I2 were spanning the breakpoint. The translocation disrupts the *KCNQ3* gene in intron 1.

To investigate whether a deletion/duplication was present in the patient microarray-CGH was carried out. A deletion on Yq11.23 (chrY: 23,540 - 26,800 kb; UCSC genome browser May 2004) was identified. This region contains numerous segmental duplications known to predispose to deletions and duplications. Similar deletions have been identified in other cohorts (Reinhard Ullmann unpublished results) and thus suggests that it is of no clinical relevance.

The most consistently suggested inheritance pattern for autism spectrum disorders (ASDs) involves several epistatic genes [2] and thus fits either an oligogenic or polygenic model of inheritance. The existence of a threshold or a point of balance has been postulated [156] where you exceed the threshold if you inherit a sufficient number of susceptibility genes from both parents. In line with this theory we identified a *de novo* truncating mutation of *KCNQ3* and the family history suggests the presence of additional susceptibility genes for mental disorders (Asperger syndrome, behavioural difficulties and recurrent depressive disorder with seasonal pattern). It is likely that there is an overlap in susceptibility factors for some mental disorders since bipolar disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) tend to segregate in the same families as ASDs, show considerable overlap in linkage intervals and some patients with ASD even have comorbid bipolar disorder, schizophrenia or ADHD [10, 13, 20].

The brain areas most consistently found to be affected in neuropathological investigations of autism are the limbic system, cerebellum and cerebral cortex [42]. Interestingly, immunohistochemical studies of *Kcnq3* in adult mice showed widespread brain localization, however, the most prominent staining was observed in the hippocampal formation, the cortex and the cerebellum as well as the reticular thalamus

and the red nucleus [157]. The intensity of the immunohistochemical staining pattern increased and the staining pattern changed during development, from a predominantly somatic staining to a gradually more dendritic/axonal staining during maturation [157].

KCNQ3 is one of five known genes encoding subunits for potassium ion channels [158]. *KCNQ3* can coassemble with *KCNQ2* and *KCNQ5* into heteromeric slowly opening and closing voltage-gated potassium channels called M-channels [158]. Several neurotransmitters and neuromodulators can influence the properties of M-channels [158]. M-channels are partially active in the range of the neuronal resting membrane potential but becomes further activated by membrane depolarization [158]. Since the M-channels are slow reacting they are not involved in the fast propagation of action potentials but rather restrain repetitive neuronal discharges that may otherwise lead to hyper-excitability [158]. The importance of this mechanism is substantiated by the identification of mutations in *KCNQ2* and *KCNQ3* in patients with dominantly inherited benign familial neonatal convulsions (BFNC) [159, 160]. BFNC is characterized by the occurrence of frequent generalized tonic-clonic seizures starting within the first days of life and spontaneously disappearing after a few weeks or months [160]. These infants experience normal growth and development but 10-16% experience a recurrence of seizures later in life [158, 161]. Even minor reductions in the M-channel activity can lead to BFNC, which is supported by the identification of a deletion of most of the *KCNQ2* gene in a patient with BFNC [162]. The translocation in our patient disrupts *KCNQ3* and thus may likewise lead to haploinsufficiency for a subunit of the M-channel. Although the translocation patient did not have the classically described neonatal convulsions he had a weak quivering during the first weeks after birth.

The phenotype observed in a transgene mouse expressing a dominant negative *KCNQ2* protein varied according to when it was expressed during development [161]. Expression of dominant negative *KCNQ2* during the first weeks of life resulted in morphological abnormalities in the hippocampus, behavioural hyperactivity and frequent seizures whereas expression during adulthood caused impairment of hippocampus-dependent memory without causing morphological changes [161]. Moreover, this experiment showed that M-channels are essential for the characteristic sub-threshold theta frequency resonance of hippocampal pyramidal cells, which is believed to be important for temporal coding and synaptic plasticity and therefore is important in sensory-motor behaviour, learning and memory [161, 163]. Accordingly, the M-channel

seems to be important for neural network excitability, postnatal brain development and cognitive performance [161]. This is interesting in concern to the translocation patient as a deficit in the integration of cognitive mechanisms has been suggested as an underlying cause of autism, schizophrenia, epilepsy and other brain-derived disorders [164]. However, even though the dominant negative KCNQ2 protein was specifically shown to attenuate mouse KCNQ2/KCNQ3-mediated currents in an *in vitro* expression system [161] it is still not known whether the observed phenotype in the dominant negative mouse is due entirely to KCNQ2/KCNQ3 M channel dysfunction or whether KCNQ2-specific functions are crucial in developing the phenotype. Thus, reduced KCNQ3 may not lead to the same phenotype. Even so, our translocation patient has an inactivating mutation of *KCNQ3* that is likely to reduce the M-current, which again may have influenced the postnatal brain development and cognitive performance and thus has contributed to the development of autism. We therefore hypothesize that *KCNQ3* is a susceptibility gene for ASD.

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5 PAPER III

FGF2 antisense gene disrupted in a male with autism spectrum disorder

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ABSTRACT

Autism spectrum disorders (ASDs) is a heterogeneous group of neurodevelopmental disorders with evidence for a strong genetic component. However, only a few susceptibility genes have been confirmed. We molecular characterized a maternally inherited translocation $t(4;16)(q27;p13.3)mat$ in a Danish, male ASD patient. The chromosome 4 breakpoint disrupts the *FGF2* (*Fibroblast growth factor 2*) *antisense* gene, *NUDT6*. Since dysregulation of the FGF signalling network has previously been implicated in the development of some psychiatric disorders and this dysregulation can partly explain how the observed male preponderance arise and how environmental factors (stress) might induce ASD in genetically vulnerable individuals we suggest *FGF2* and *NUDT6* as susceptibility genes for ASD.

KEYWORDS

Autism spectrum disorder (ASD), translocation, Chromosome 4q27, *NUDT6*, *FGF2*,

INTRODUCTION

Autism spectrum disorders (ASDs) are characterized by impairments in reciprocal social interaction and communication as well as by stereotypic behaviour or interests. ASDs are considered to be neurodevelopmental disorders with early childhood onset and a lifelong persistence. The prevalence of ASDs is estimated to be between 3 - 9 in 1000 [5] with a male to female ratio of 4:1 [2]. ASDs are amongst the most heritable complex disorders with a monozygotic concordance rate of 60-90% compared to a dizygotic concordance rate of approximately 5% [1, 2]. However, since the monozygotic concordance rate is not 100% environmental factors also play part in the development of at least some ASD cases. The mode of inheritance is not known but it is likely that the huge variation in phenotype reflects a huge genetic heterogeneity. The most commonly suggested genetic model involves several epistatic genes but whether these genes are always the same or change between families is not known [2]. Despite the apparent robust genetic basis of ASDs no susceptibility genes or pathophysiological mechanisms have been unambiguously elucidated.

Recent evidence suggest that dysregulation of the FGF signalling network can be involved in the development of some psychiatric disorders [30, 165]. In addition, a LOD score of 2,65 at marker D4S3250 close to *NUDT6* and *FGF2* was reported in a

genome wide screen for ASDs [104], which further suggests a role for *FGF2* in the development of ASDs. In line with this theory we report an ASD patient with a disruption of the *antisense FGF2* gene, *NUDT6*, by a maternally inherited translocation.

MATERIALS AND METHODS

Patient

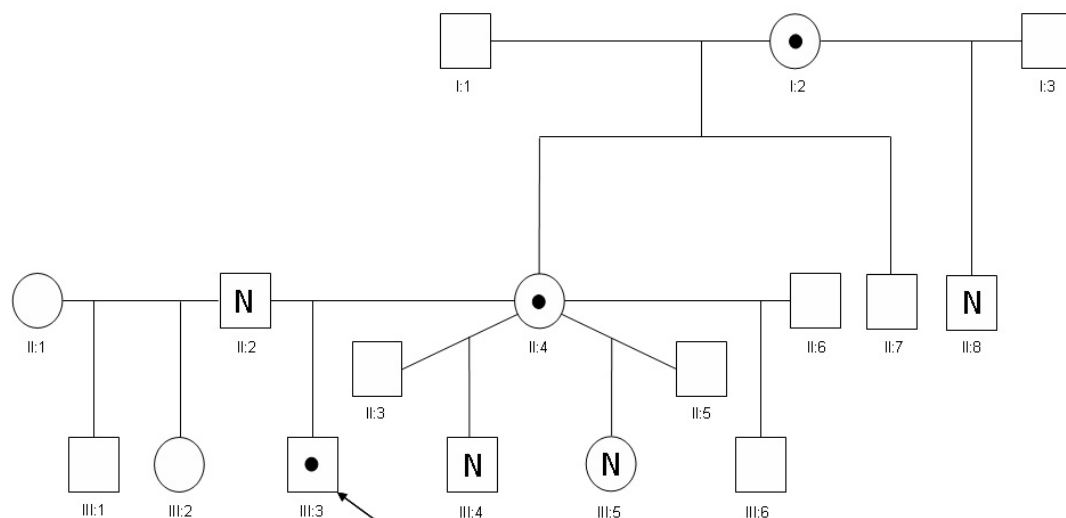


Figure 1. Pedigree showing translocation carriers with a dot and examined non-carriers with an N. Unmarked individuals have not been cytogenetically examined.

A 22 year old Danish man carries a translocation $t(4;16)(q27;p13.3)_{mat}$. An Autism Diagnostic Interview (ADI-R) as well as an Autism Diagnostic Observation Scheme (ADOS) was used to confirm that the autism spectrum disorder (ASD) diagnosis was in accordance with ICD-10 criteria. He was 17,5 years of age when the described tests were conducted. He can not read or write. A WISC test revealed language skills corresponding to a 9 - 9,5 year old and performance skills corresponding to a 6 to 12 year old whereas, the Peabody Picture Vocabulary Test (PPVT) revealed a passive vocabulary corresponding to a 17,7 year old. Pregnancy and delivery were without complications. To provide the needed help, the proband and his mother lived first in an institution and afterwards in a foster family. When the proband was approximately 1,5 years old he was transferred to a home for infants where he lived until the age 5 where he moved to a foster home. Accordingly, little is known about the developmental milestones within his first years. At the age of 12 he moved to a residential home run by

his foster parents. This institution houses three children or youngsters. The proband has attended a school for children with learning difficulties. He recently moved to his own flat in a house shared by 29 additional youngsters that all require supervision by social workers.

The mother (II:4; figure 1) is a carrier of the same translocation as the proband. She works as an IT-supporter and has four children with four different men. Only the youngest child lives with the mother (and father). The mother was given the custody of her youngest child but under supervision. The maternal grandmother (I:2; figure 1) also carries the translocation and is phenotypically normal. The father (II:1; figure 1) can not read or write and has impairment of speech. He has attended Danish lessons at a school for mentally retarded individuals. He has two children (younger than the proband) with another woman. Both of these children have attended a day care centre and a school for children with special needs where they have received remedial teaching.

The National Ethics Committees and the Danish Data Protection Agency approved the study, and informed consent was obtained.

Fluorescence *in situ* hybridization (FISH)

Metaphase chromosomes were prepared from peripheral blood lymphocytes and the karyotype of the patient was determined by G-banding. Fluorescence in situ hybridization (FISH) was performed using bacterial artificial chromosome (BAC) clones from the RPCI-11 library and standard protocols. The BAC clones were obtained from the MCN reference center at Max Planck Institute for Molecular Genetics, Berlin (<http://www.molgen.mpg.de/~cytogen/>) or the Wellcome Trust Sanger Institute, Cambridge: (http://www.sanger.ac.uk/cgi-bin/software/archives/new_clone_login.cgi). 250 ng BAC DNA was biotin-14-dATP labeled by nick translation and hybridized to patient metaphase chromosomes. Signals were visualized using avidin-FITC detection system and chromosomes were counterstained with DAPI. Signals were investigated using a Leica DMRB epifluorescence microscope equipped with a Sensys 1400 CCD camera (photometrics) and an IPLab Spectrum imaging software (Vysis).

Southern blot

The location of restriction sites and sizes of restriction fragments were identified by the NEBcutter program: <http://tools.neb.com/NEBcutter2/index.php>. Templates for probes were made by cloning PCR-products (primer information table 1) using a TOPO TA cloning[®] kit (Invitrogen, Carlsbad, CA, USA) and subsequently re-amplifying the PCR products using a biotinylated M13 primer (TAG Copenhagen, Copenhagen, DK). The biotinylated strand was retained using streptavidin-coated DynaBeads (Dyna, Oslo, Norway) and the complementary strand was resynthesized in the presence of α -³²P-dATP and modified dCTP as described in the Strip-EZ[™] DNA kit (Ambion, Ausin, TX, USA). The radioactive strand was isolated, dissolved in hybridization solution (Ambion, Ausin, TX, USA) and used as probe. Hybond N filters (Amersham Biosciences, NJ, USA) were used. The filters were stripped as described in Strip-EZ[™] DNA kit.

Primer name		Primer sequence	Size of PCR product
NUDT6 cDNA	Forward	5' GCGATGCTTGCCCGAACCTAC 3'	608 bp
	Reverse	5' TTTGTGTGCTGTTGCCGAATACTCA 3'	
NUDT6 exon 1	Forward	5' CCATTTACCCAACCGCCTTAGAGA 3'	476 bp
	Reverse	5' CATTTGGGCAACGGACGAATTAAGC 3'	

Table1. PCR primers for southern blot probes.

Micro-array based CGH

Array CGH with a whole genome 32K BAC array was carried out as described in [115]. and detailed protocols can be downloaded from:

http://www.molgen.mpg.de/~abt_rop/molecular_cytogenetics/.

Data analysis and visualization was done in CGHPRO [166].

Sequencing

Mutation analysis of the *FGF2* and *NUDT6* genes was carried out by direct sequencing in the proband and the maternal grandmother. We analyzed all coding exons and splice sites corresponding to clones NM002006 and NM007083. The sequencing reactions were carried out by MacroGen Inc in Korea (<http://www.macrogen.com/>) and ChromasPro version 1.33 (Technelysium Pty Ltd, Australia) was used to analyze the data. Primers used are listed in table 2.

Primer name		Primer sequence	Size of PCR product
FGF2 exon1	Forward	5' CCTCTCCCCCGCCCCGACTG 3'	803 bp
	Reverse	5' TGGAGGGGAGAGAGCGGGCGAGAAC 3'	
FGF2 exon2	Forward	5' GACACAGGAGCGACAAGGA 3'	470 bp
	Reverse	5' TTACAGGGTGACTATGACGATGA 3'	
FGF2 exon3	Forward	5' GCCCATTGAATCTTGTAGTTTGA 3'	509 bp
	Reverse	5' TTTTCTTTACTGGGACAATGGTTACATA 3'	
NUDT6 exon1	Forward	5' CCATTTCACCCAACCGCCTTAGAGA 3'	476 bp
	Reverse	5' CATTTGGGCAACGGACGAATTAAGC 3'	
NUDT6 exon2	Forward	5' AACAAAGCAAGAAATTGGGTGAACGTCTA 3'	549 bp
	Reverse	5' ACATCTGTTACGTGCCAAGCATTGTC 3'	
NUDT6 exon3	Forward	5' CCTGGCACAGAGTAGAAACTCATTGAA 3'	467 bp
	Reverse	5' AGAGAGTGTGGGAGGAGATTAAGGTGA 3'	
NUDT6 exon4	Forward	5' TTAATTTCTCAACATTTTAAAGCCAAT 3'	260 bp
	Reverse	5' GGGAGAAAGGATGGAAGCTAGAA 3'	
NUDT6 exon5	Forward	5' CTATTTCTTATGTCATTCGTT 3'	549 bp
	Reverse	5' GAGCCTTTACATAATGCTAC 3'	

Table 2. Primers used for sequencing of FGF2 and NUDT6.

RESULTS

Fluorescence *in situ* hybridization (FISH)

BAC clones RP11-951C12 and RP11-698H1 on chromosome 16 were both distal to the breakpoint whereas the overlapping cosmid clone 352F10 (AC005361) was proximal. Thus, the breakpoint on chromosome 16 was most likely located in the gene free area between serine protease 33 (*PRSS33*) and serine protease 21 (*PRSS21*).

On chromosome 4 BAC clone RP11-379D14 was proximal, RP11-636M7 was distal and RP11-170N16 (AC021205) was spanning the breakpoint which narrowed down the breakpoint region to approximately 60 kb encompassing *Nucleoside Diphosphate-Linked Moiety X Motif 6* (*NUDT6*) as well as part of *Fibroblast Growth Factor 2* (*FGF2*) and *spermatogenesis associated factor SPAF5* (*SPATA5*).

Southern blot

DNA from the proband and two controls was digested with *BtgI*, *BanI*, *Asel*, *DraIII*, *EcoRI* and hybridized with a probe specific to exon 1 (Figure 3) of *NUDT6* whereas the DNA digested with *AflIII* were hybridized with a *NUDT6* cDNA probe. The *AflIII* band of 1999 bp corresponds to the restriction fragment containing exon 1 of *NUDT6* whereas the

band of 2449 bp corresponds to the fragment encompassing exon 2. One restriction site of *BtgI* is located within the probe used for southern blot. Because of this 142 bp of the 475 bp probe recognizes the restriction fragment of 15338 bp whereas 333 bp of the probe recognizes the restriction fragment of 2108 bp. By adding all southern results (Figure 2) the breakpoint on chromosome 4 was narrowed down to 812 bp within intron 1 of *NUDT6* (Figure 3).

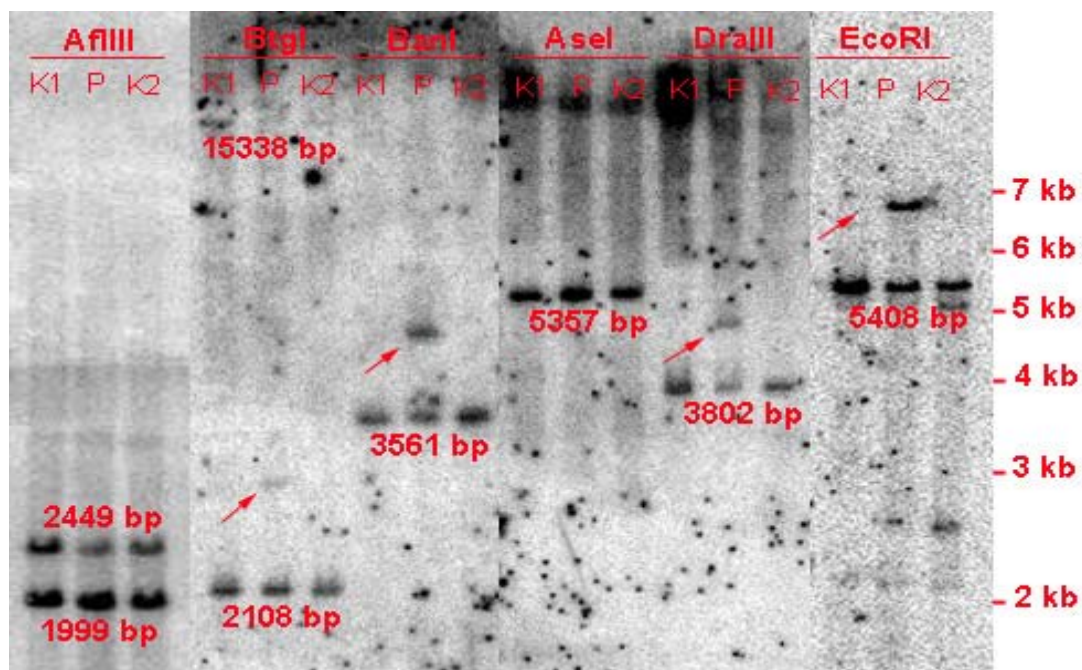


Figure 2. Southern blot results. K1 and K2 are controls, P is the patient. Additional bands in the patient lane (corresponding to restriction fragments encompassing the translocation breakpoint) are marked with an arrow.

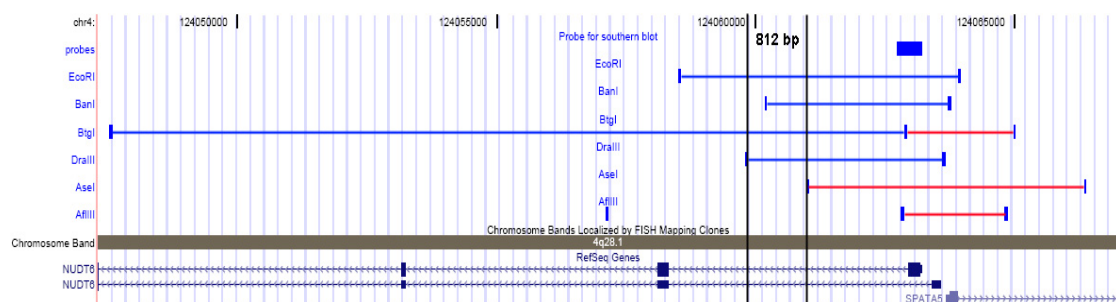


Figure 3. Screen plot from the UCSC genome browser Mar.2006 showing southern blot enzyme restriction sites and annotated genes within the breakpoint region. The translocation breakpoint was located within restriction fragments highlighted in blue but not in fragments highlighted in red.

Micro-array based CGH

Apart from known DNA copy number variants as listed in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>), no other DNA copy number changes were detected by means of array CGH.

Sequencing

No changes were identified in *FGF2* in the proband and the maternal grandmother. Both the grandmother and the proband were homozygous (C/C) for SNP rs13134412 and (G/G) for SNP rs12648093 in *NUDT6*. Moreover, the grandmother was heterozygous (A/G) for SNP rs1048201.

DISCUSSION

The most consistently suggested inheritance pattern for ASDs involves transmission of several epistatic genes [2] and thus fits either an oligogenic or polygenic model of inheritance. The existence of a threshold or point of balance has been postulated [156] meaning that a threshold is reached when a sufficient number of susceptibility genes are inherited from both parents. According to this theory a full ASD phenotype in the parents of the proband would not be expected even though they carry some of the ASD susceptibility genes. It is, however, common to find the conceptually same traits in parents and siblings of ASD patients but in a milder form that is not necessarily associated with difficulties in functioning and might even be associated with high achievement [2]. This broader autism phenotype is characterized by social reticence, communication difficulties, preference for routines and difficulty with change [2]. In line with this theory the disruption of *NUDT6* on chromosome 4 by the maternally inherited translocation might contribute to the ASD phenotype in the proband even though the female translocation carriers do not have an ASD diagnosis. In addition, delayed onset of speech and difficulty with reading is a common finding in family members of ASD individuals [2]. In agreement with this observation the father of the proband is unable to read and write and has impairment of speech. Hence, it is likely that genetic factors from both the mother and the father were involved in the development of ASD in the proband. However, sequencing of *NUDT6* and *FGF2* in the proband and the maternal grandmother showed that the paternal contribution to the ASD phenotype in the proband is not mediated by mutations in *NUDT6* and *FGF2*.

On chromosome 16, the breakpoint is most likely located between *PRSS33* (*serine protease 33*) and *PRSS21* (*serine protease 21*). *PRSS33* is predominantly expressed in spleen, stomach, retina, ovary and leukocytes and it is suggested to be associated with macrophages [167] whereas *PRSS21* is predominantly expressed in lung, pancreas, spleen, thymus, prostate and testis [168]. Accordingly, these genes are not likely susceptibility genes for ASD.

NUDT6 encodes a protein of the nudix family of phosphohydrolases (MutT family), whose function in mammalian cells is currently unknown but the *E.coli* ortholog reduces the spontaneous mutation rate 100-1000 fold [169]. *NUDT6* is also known as the *FGF2 antisense (FGF2AS)* gene because it is transcribed from the opposite DNA strand of the *FGF2* gene and overlaps the 3' end of *FGF2* [170]. Moreover, *FGF2* and *NUDT6* are co-ordinately transcribed on a tissue-specific and developmentally regulated basis [171]. In addition, an inverse association of *NUDT6* and *FGF2* mRNA level has been observed in a variety of tissues across several species and forced over-expression of *NUDT6* mRNA in stably transfected cells effectively suppresses *FGF2* expression [170, 172]. It has therefore been suggested that *NUDT6* mRNA is implicated in the posttranscriptional regulation of *FGF2* mRNA expression either by nuclear retention or rapid degradation of the mRNA or interference with mRNA processing, transport, translation or stability [172]. There are no identified copy number variations of the chromosomal region encompassing *NUDT6* and *FGF2* in the database of genomic variants (<http://projects.tcag.ca/variation/>) which implies that dosage of these genes is important.

FGF2 is highly expressed in brain and is a member of the heparin-binding growth factor superfamily that comprises 21 additional human FGF ligands [173, 174]. Together with the five identified FGF receptors (FGFR) they constitute the FGF signaling system that is dynamically organized in spatial and temporal expression patterns [175]. *FGF2* plays versatile biological roles. At an early developmental time point *FGF2* functions as a mitogen for different neuronal precursor cells and as a differentiating factor for calbindin-expressing hippocampal neurons [173]. Likewise, *FGF2* induces proliferation of neural progenitor cells in the hippocampus and the subventricular zone in the adult brain [173]. The mitogenic function of *FGF2* is substantiated by knock out mice that display abnormal cytoarchitecture and fewer mature neurons and glia cells in the neocortex, particularly in the frontal motor sensory areas [176, 177]. Furthermore, *FGF2*

seems to be important in cell positioning in the developing neocortex since a fraction of postmitotic neurons fail to reach their target layer in FGF2^{-/-} mice [178]. The knock out mice are otherwise viable, fertile and phenotypically indistinguishable from normal mice [176]. In addition, FGF2 can promote long term potentiation (LTP) in hippocampal slices, induce synaptogenesis in cultured rat embryonic hippocampal neurons and is particularly effective in promoting branching of axons in cultured cortical neurons [179-182]. Moreover, spatial learning and physical activity increases the expression of FGF2 mRNA in rat hippocampus [180]. Accordingly FGF2 is suggested to be involved in learning and memory.

Of particular interest in concern to the translocation patient is, that FGF2 function as a trophic and neuroprotective factor, preventing neuronal death within lesioned brain regions after ischemia and of cholinergic neurons of the hippocampus and dopaminergic nigrostriatal neurons after fiber transection or chemical injury [173, 183]. Similarly, FGF2 expression is increased following acute restraint stress [30]. Because FGF2 is neuroprotective it is possible that reduced expression can increase the vulnerability of selected neuronal populations. This has been proposed as an underlying cause of some psychiatric disorders [184]. In line with this theory, a recent study identified dysregulation of multiple FGF/FGFR transcripts in postmortem brains from major depressed individuals and to a lesser degree in schizophrenic individuals [175]. In addition, this signaling system is modulated by treatment with psychotropic drugs (serotonin reuptake inhibitors, diazepam, clozapine) [165]. The level of FGF2 transcripts are specifically reduced in the fronto-cortical area and several limbic system structures in post mortem brains and increased in hippocampus after administration of the above mentioned drugs [175, 184]. It is likely that there is an overlap in susceptibility factors for at least some mental disorders since bipolar disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) tend to segregate in the same families as ASDs, show considerable overlap in linkage intervals and some patients with ASD have comorbid major depression, bipolar disorder, anxiety disorder, schizophrenia or ADHD [10, 13, 20, 185, 186]. It is therefore conceivable that dysregulation of the FGF system might also be involved in the development of ASD in some patients. This might at least be part of the explanation for the phenotype in the translocation patient described above. He only has one functional copy of *NUDT6* which possibly causes misregulation of FGF2 which would render him more vulnerable to stress. In addition, adverse events during late pregnancy

and early postnatal life in otherwise normal rats can produce permanent neurobiological and behavioural abnormalities later in life, including increased responsiveness to stress [30]. Some of these effects are male-specific [30] which may in part explain the observed difference in phenotype of the proband and the two female translocation carriers in the family (Figure 1; I:2, II:3). Hence, our proposal of *FGF2* and *NUDT6* as candidate susceptibility genes for ASD may have relevance for both the observed preponderance of males in ASD and for how environmental factors (stress) could elicit ASD in genetically predisposed individuals.

ACKNOWLEDGEMENTS

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6 PAPER IV

Indication of abnormal synapse formation in male twins with autism spectrum disorder

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ABSTRACT

Autism Spectrum disorders (ASDs) are heterogeneous disorders with unknown aetiology. Even though ASDs are suggested to be amongst the most heritable complex disorders only a few susceptibility genes have been confirmed. In a Swedish, male monozygotic twin couple diagnosed within the ASD spectrum we identified a maternally inherited inv(10) and a paternally inherited t(9;18)(p22;q21.1). We showed that the inverted chromosome 10 is a benign variant chromosome with no apparent phenotypical implications (**paper V**) [187]. Further analyses of the chromosomes of the patients revealed several genomic changes that might have contributed to the development of ASD. The translocation breakpoint at 18q21 disrupts *ZBTB7C* (*zinc finger and BTB containing 7C gene*). We showed that *ZBTB7C* was expressed in many brain regions, including the limbic system, cerebellum and cerebral cortex, regions known to be affected in ASDs. Furthermore, analyses with array-CGH revealed a maternally inherited deletion on chromosome 8q22 encompassing the *RIM2* (*Rab3A-interacting molecule 2*) and *BAALC* (*Brain and Acute Leukemia Gene, Cytoplasmic*) genes as well as a duplication on 5p14 including exons 1 and 2 of the *CDH9* (*cadherin 9*) gene in the twins. The corresponding protein products function at synapses. In addition, a paternally inherited nucleotide change in an ultra conserved sequence (UCS) located in the conserved regulatory landscape at *BRUNOL4* (*bruno-like 4 RNA binding protein*) was identified. Since UCSs are suggested to be gene regulatory elements such a mutation might alter the expression of *BRUNOL4*. Like *ZBTB7C*, *BRUNOL4* is expressed in the limbic system, cerebellum and cerebral cortex and might accordingly be involved in the development of ASDs. The common functional theme of the candidate susceptibility genes identified in this study suggests that synaptic dysfunction is the basis for ASD in the presented twins. Moreover, the identification of accumulated genetic alterations inherited from both parents in the patients presented here supports the complex genetic basis of ASDs, which has most commonly been suggested. Furthermore, the study supports that detailed molecular dissection of patients with multiple inherited chromosomal rearrangements may reveal information about susceptibility genes for ASDs.

KEYWORDS

Autism, translocation, inversion, deletion, duplication, chromosome 18, chromosome 8, chromosome 5, chromosome 10, synapse, additive effect.

INTRODUCTION

ASDs are neurodevelopmental disorders with onset in early childhood. They are characterized by impairment in reciprocal social interaction and communication accompanied by stereotypic behaviours or interests [2]. The severity of the qualitative impairments can vary greatly between individuals. The prevalence of childhood autism is estimated to be between 10 and 60 in 10000 [5-7] with a male to female ratio of 4:1 [2]. Childhood autism is amongst the most heritable complex disorders with a monozygotic concordance rate of 60-90% compared to a dizygotic concordance rate of approximately 3% [1, 2]. The mode of inheritance for ASDs is not known but it is likely that the large variation in phenotype reflects a large genetic heterogeneity. The most common genetic model suggests involvement of several epistatic genes but whether these genes are always the same or whether different combinations of genes from a larger pool of susceptibility genes can be involved is not known [2]. Despite considerable ongoing efforts towards identification of putative susceptibility genes for ASDs, only a few genes have been reproducibly shown to be associated [41]. Some of the proteins encoded by these genes are involved in synaptogenesis and/or maintenance of the synapses, which suggests that alterations of these biological processes might be involved in the development of at least some cases of ASDs.

METHODS

Patients

The patients are 13 year old Swedish, male twins diagnosed within the ASD spectrum. The diagnosis was made when the twins were three years old. The pregnancy and delivery was normal but a cupping glass was used at the delivery of patient 1. At birth patient 1 had a weight of 3015 g, a length of 48 cm and a head circumference of 34 cm. Apgar scores were 6 after 1 minute, 9 after 5 minutes and 9 after 10 minutes. The birth weight of patient 2 was 2925 g, birth length was 47 cm and head circumference at birth was 33 cm. Apgar scores were 9 after 1 minute and 10 after 5 minutes. Delayed motor development was noted at 10 month of age for both twins. Both twins could crawl at 16

months of age and walk at 52 months. The twins do not speak but can to some degree communicate by means of pictures. No dysmorphic features are noted in the twins. The older sister and the parents are phenotypically normal and the parents are unrelated. No mental handicaps have previously been diagnosed in the family. The initial cytogenetically determined karyotype of the twins was:

46,XY,t(9;18)(p22;q21.1)pat,inv(10)(p11.2q21.2)mat.

The National Ethics Committee and the Danish Data Protection Agency approved the study, and informed consent was obtained.

Test of monozygosity

7 markers (D2S2393, D6S1691, D10S213, D12S80, D14S80, D15S1048, D20S196) were typed by standard methods. Primers were radioactively end labelled, PCR was carried out under standard conditions and bands were separated by electrophoresis under denaturing conditions on polyacrylamide gels, and autoradiography was carried out.

Cytogenetic analyses and Fluorescence in situ hybridisation (FISH)

Metaphase chromosomes were prepared from peripheral blood lymphocytes and the karyotypes were determined by G-banding. FISH was performed using bacterial artificial chromosome (BAC) clones from the RPCI-11 library and standard protocols. The BAC clones were obtained from the MCN reference centre at the Max Planck Institute for Molecular Genetics, Berlin (<http://www.molgen.mpg.de/~cytogen/>) or the Wellcome Trust Sanger Institute, Cambridge (http://www.sanger.ac.uk/cgi-bin/software/archives/new_clone_login.cgi).

Southern Blot

The location of restriction enzyme sites for EcoRI, SacI and ApaI and sizes of restriction fragments were identified by the NEBcutter program:

<http://tools.neb.com/NEBcutter2/index.php>. Probes were prepared by cloning PCR-products using a TOPO TA cloning[®] kit (Invitrogen, Carlsbad, CA, USA) and subsequently re-amplifying the PCR products using a biotinylated M13 primer (TAG Copenhagen, Copenhagen, DK). The biotinylated strand was retained using streptavidin-coated DynaBeads (Dyna, Oslo, Norway) and the complementary strand was

resynthesized in the presence of α -³²P-dATP and modified dCTP as described in the Strip-EZ™ DNA kit (Ambion, Ausin, TX, USA). The radioactive strand was isolated, dissolved in hybridization solution (Ambion, Ausin, TX, USA) and used as probe. Sequences of all primers are listed in supplementary table 1.

Microarray-based CGH

Array-based comparative genome hybridization (array-CGH) with a whole genome 32K BAC array was performed for patient 1 and both parents. The method is described in [115].

Denaturing high performance liquid chromatography (dHPLC)

Mutation analysis of the five ultra conserved sequences (UCS) close to *BRUNOL4* included the sequences originally defined by Bejerano [118]: uc.429: chr18:32,732,529-32,732,765; uc.430: chr18:33,315,638-33,315,849; uc.431: chr18:33,430,588-33,430,816; uc.432: chr18:33,816,920-33,817,129; uc.433: chr18:34,315,620-34,315,824 (position refers to hg16). Primer sequences are listed in supplementary table 2. Melting temperature (T_m) was predicted based on the fragment sequence by using: <http://insertion.stanford.edu/melt.html>. Fragments were analyzed using standard operating procedure of a Varian Helix™ DHPLC analysis system with a Helix™ DHPLC column (Varian Inc. CA., USA).

Sequencing analyses

The sequencing reactions of UCSs were carried out by Macrogen Inc in Korea (<http://www.macrogen.com/>) and ChromasPro version 1.33 (Technelysium Pty Ltd, Australia) was used to analyze the data. Sequencing of the two identified coding exons of *ZBTB7C* was carried out using a Thermo sequenase [P³³] Terminator Cycle Sequencing Kit (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions. Sequencing gels were analyzed using a Storm™ 860 Gel and Blot Imaging System (Amersham Biosciences). Primer sequences for UCSs and *ZBTB7C* are listed in supplementary table 2 and 4 respectively.

Real-time quantitative PCR analysis (Q-PCR) of ZBTB7C

Messenger RNA and total RNA from 34 tissues (supplementary table 7 and 8)(Clontech, CA, USA) were DNase treated before cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) was carried out according to manufacturer's instructions. Synthesized cDNA was checked for DNA contamination by PCR with three primer pairs (supplementary table 3). Q-PCR analysis was carried out on a DNA Engine Opticon 2 (Bio-Rad, Göteborg, Sweden) using LightCycler FastStart DNA Master^{PLUS} SYBR GreenI (Roche, Hvidovre, Denmark). From 12 analyzed housekeeping genes, six were selected for normalization by using the BestKeeper software [116]. Primers used are listed in supplementary table 6.

mRNA in situ hybridization

Coronal cryostat sections of the mouse brain, 12 µm in thickness, were cut and mounted on Superfrost Plus[®] slides. The sections were hybridized as previously described [117] with three 38-mer ³⁵S-labeled oligonucleotide probes complementary to mRNA encoding ZBTB7C. An oligonucleotide probe was used for sense control (see supplementary table 5 for probe information). Sections were exposed to an X-ray film for 1 to 2 weeks.

RESULTS

Test of monozygosity

Zygosity was evaluated by genetic analysis of 7 highly polymorphic markers located at 7 different chromosomes.

Marker	Mother	Father	Twin 1	Twin 2	L _i
D2S2393	1, 2	1, 2	2, 2	2, 2	0.25
D6S1691	1, 3	2, 3	2, 3	2, 3	0.25
D10S213	1, 3	1, 2	1, 3	1, 3	0.25
D12S80	1, 2	1, 2	1, 1	1, 1	0.25
D14S80	1, 3	2, 4	3, 4	3, 4	0.25
D15S1048	1, 2	2, 3	1, 3	1, 3	0.25
D20S196	1, 3	2, 4	1, 2	1, 2	0.25

Table 1. Marker analysis in a pair of twins and their parents. The number in the DNA genotypes of the individuals represent the different alleles at a specific locus. L_i is the likelihood ratio of conditional probabilities of dizygotic and monozygotic twins to be identical at a given locus.

The probability of monozygosity, P(MZ), was calculated according to Bayers principle of conditional probabilities: $P(MZ) = 1/(1+(Q*L))$ [188]. Q is the ratio of dizygotic and

monozygotic twins in the population ($Q = 2.33$ in European populations) and L is the likelihood ratio of conditional probabilities of dizygotic and monozygotic twins to be identical at a given combination of genetic markers. L is calculated by multiplying the L_i values. The probability of monozygosity was 0.99994.

Fluorescence *in situ* hybridization (FISH)

FISH was carried out to characterize the translocation and inversion breakpoints. The inversion of chromosome 10 was a common variant most likely with no phenotypical relevance as published by our group (**paper V**) [187].

On chromosome 18 the BAC clone RP11-8K24 was proximal, RP11-246E12 was distal and RP11-656P10 spanned the breakpoint. The breakpoint region on chromosome 18 was narrowed down to 26.8 kb within the overlap of the proximal and distal BAC (chr18:43,796,962-43,823,759; NCBI35; HG17). *ZBTB7C* is the only annotated gene within this region.

On chromosome 9 the BAC clone RP11-380P16 and cosmid clone G248P87527B3 were distal, BAC clone RP11-1057A18 was proximal and the BAC clones RP11-158I17 and RP11-956G20 as well as the cosmid clone G248P86061B3 were spanning the breakpoint. The breakpoint region on chromosome 9 was narrowed down to approximately 40.6 kb (chr9:21,351,027-21,391,627; NCBI35; HG17) including two interferon α genes: *IFNA2* and *IFNA13*.

FISH was carried out to affirm the BAC array-CGH results. FISH with BAC clone RP11-318M2 confirmed the presence of the 8q22 deletion in both twins as well as in the mother. FISH with BAC clone RP11-184A13 located within the duplicated region on 5p14 revealed signals only on the two short arms of chromosome 5, which therefore excludes that the duplication has been inserted into a different chromosome.

Southern Blot

The breakpoint on chromosome 18 was narrowed down by Southern blot analysis to 1165 bp within exon 2 of *ZBTB7C* (figure 1). Position of the probe identifying the junction fragment is at: chr18:43,820,703-43,821,065 (NCBI35; HG17).

Enzyme	Restriction sites (NCBI35; HG17)	Restriction fragment size
EcoRI	chr18:43,818,423–43,823,597	5174 bp
ApaLI	chr18:43,820,295–43,823,676	3381 bp
SacI	chr18:43,816,879–43,821,459	4580 bp

Table 2. Position of restriction sites and sizes of normal restriction fragments recognized by the probe.

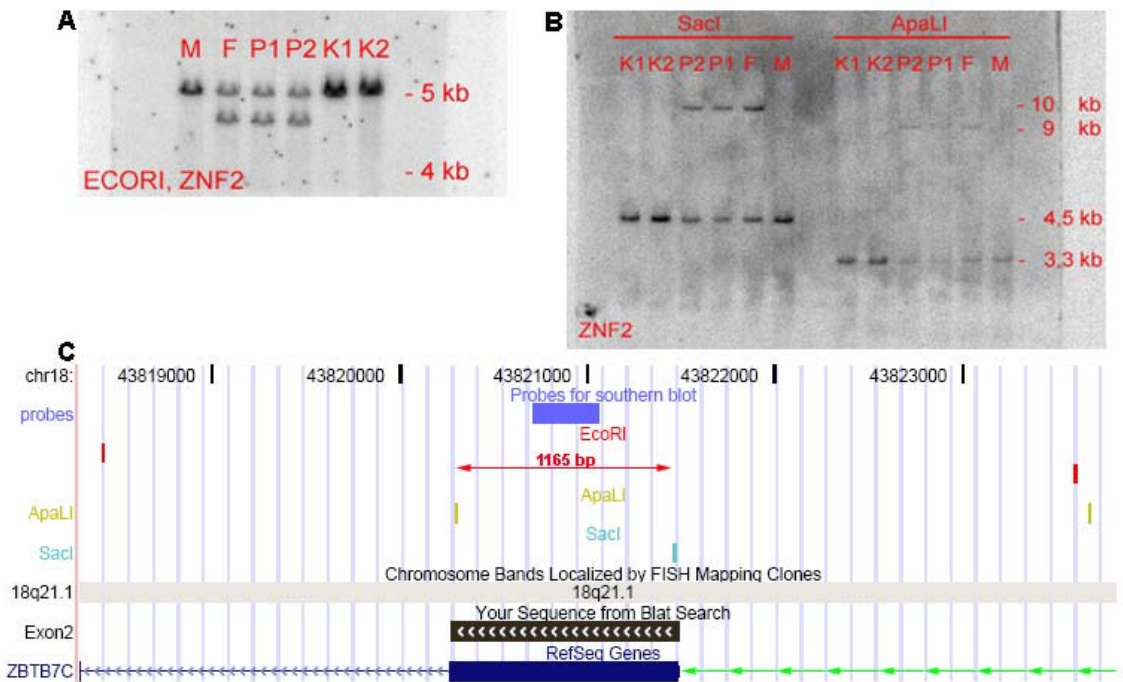


Figure 1. **A:** Southern blot hybridization. DNA digested with EcoRI. The probe detects a 5 kb normal fragment in all individuals, and an approximately 4.8 kb junction fragment in the father (F) and both patients (P1 and P2). **B:** Southern blot hybridization. DNA digested with SacI and ApaLI. Following the SacI digestion, the probe detects a 4.5 kb normal fragment in all individuals and an approximately 10 kb junction fragment in the father and both patients. Likewise, for the ApaLI digestion, the probe detects a normal fragment of 3.3 kb in all individuals and an approximately 9 kb junction fragment in the father and both patients. **C:** The physical map of the genomic region at the 18q21.1 breakpoint. Restriction sites for EcoRI, ApaLI and SacI as well as the probe identifying the southern blot junction fragment on chromosome 18 by Southern blot analysis are shown. The breakpoint is narrowed down to 1165 bp within exon 2 of ZBTB7C between the ApaLI restriction site (chr18:43,820,295) and the SacI restriction site (chr18:43,821,459). The green arrows indicate that we have identified a non-coding exon 1 that is not depicted in the screenplot from the UCSC genome browser May2004. M = mother, K1 = control 1, K2 = control 2.

Array CGH

Array-based Comparative Genome Hybridization (array-CGH) was performed on DNA from patient 1. A deletion of approximately 931 kb on 8q22 (chr8:104,04 -104,97 Mb; NCBI35; HG17) comprising 7 RefSeq genes, as well as a duplication of 875 kb on 5p14.1 (chr5:26,95 - 27,83 Mb; NCBI35; HG17) comprising exons 1 and 2 of *CDH9* was identified. Array CGH revealed that the duplication on 5p14 was inherited from the mother.

Following array-CGH the karyotype of the patients were thus established as: 46,XY,t(9;18)(p22;q21.1)pat,inv(10)(p11.2q21.2)mat,
arr cgh 5p14.1(RP11-349J03->RP11-271D15)x3, 8q22.3(RP11-754L23->RP11-739L19)x1

Mutation screening of UCSs

BRUNOL4 and two out of the five ultra conserved sequences (UCSs) located close by this gene is deleted in another ASD patient examined in our laboratory. Accordingly, we screened the five UCSs for mutations in 157 ASD patients including the twins presented here. A paternally inherited C>A nucleotide change (ss67005820) was identified in the twins in uc.432 located 5' to *BRUNOL4*. This nucleotide is conserved in mouse, rat, rabbit, dog, armadillo, elephant, opossum, and chicken. The nucleotide change was not identified in 167 controls.

ZBTB7C

We designed several intron-spanning primers for reverse transcriptase PCR (RT-PCR) to ascertain that *ZBTB7C* is expressed in the brain and to establish its genomic organization. The EST clone fs01a03.y1 was obtained from the Wellcome Trust Sanger Institute and sequenced. The sequence of this clone agreed partly with our RT-PCR findings and the clone annotated in the UCSC genome browser (NM_001039360), however we identified a noncoding exon 1 (EF120357).

The two coding exons were screened for mutations in the twins and the parents. The twins, the mother and a control were all heterozygous (G/A) for SNP ss68362639 within exon 2 of *ZBTB7C*. The mother was heterozygous for two more SNPs within exon 2 of *ZBTB7C* (G/A; ss68362638) (T/C; ss68362640) whereas the control was homozygous (T/T; ss68362640) and the twins were homozygous (C/C; ss68362640) for one of the SNPs.

Q-PCR analysis of ZBTB7C

The observed expression of *ZBTB7C* was not high in any of the tissues examined. However, highest expression was observed in brain but also testis, prostate, lung, trachea, pancreas and kidney showed some expression of the gene (Supplementary table 2).

RNA in situ hybridization of mouse *zbtb7c*

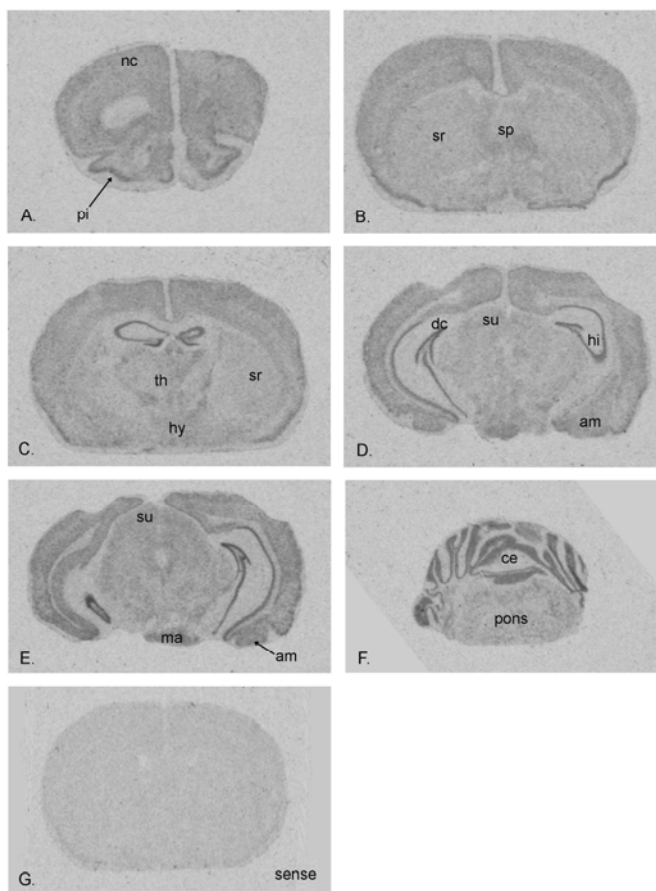


Figure 3. *In situ* hybridization for mRNA transcript of *zbtb7c* on coronal sections of the mouse brain. The montage shows images on x-ray films of hybridized coronal sections from rostral to caudal levels (A-F). G: Control. A coronal section of the forebrain, hybridized with a sense probe. am = amygdale; ce = cerebellum; de = dentate gyrus; hy = hypothalamus; hi = hippocampus; ma = mamillary nuclei; nc = neocortex; pi = piriform cortex; sp = septum; sr = striatum; su = superior colliculus; th = thalamus. The strongest signal was observed in cerebellum. Less intense signals were observed in the neocortex, amygdala, thalamus and piriform cortex.

A moderate level of *zbtb7c* gene expression was present in the forebrain, brain stem and cerebellum. The strongest signal was observed in the granular layers of cerebellum and the dentate gyrus, and the pyramidal cell layer of the cerebellum. Less intense signals were observed in the piriform cortex, the neocortex, the amygdaloid complex, the intralaminar and reticular nuclei in the thalamic complex. The mammillary nuclei also expressed a moderate signal.

DISCUSSION

In a Swedish, male monozygotic twin couple diagnosed within the ASD spectrum we identified a maternally inherited inversion *inv*(10)(p11.2q21.2) and a paternally inherited translocation *t*(9;18)(p22;q21.1). Both inversion breakpoints as well as the chromosome 9 breakpoint of the translocation were apparently located within or close to suggestive linkage intervals identified in autism genome screens [103, 107, 108]. The other translocation breakpoint in chromosome 18q21 lies within a region that was apparently deleted in a patient with mild to profound mental retardation [109]. Accordingly, we decided to molecularly examine all four chromosomal rearrangement breakpoints as they might reveal susceptibility genes for ASDs.

Two interferon genes, *IFNA2* and *IFNA13*, were located within the translocation breakpoint region on 9p21.3. These are not obvious susceptibility genes for ASDs. In contrast, we found that *ZBTB7C* was truncated by the 18q21 translocation breakpoint. *ZBTB7C*, first described in 1998 by Reuter et al as a putative cervical tumour suppressor [189], contains an N-terminal BTB/POZ domain (amino acid 34-131) that is known to mediate protein-protein interaction and four DNA-binding C₂H₂ zinc-finger domains [189, 190]. The expression pattern of *zbtb7c* in mouse brain overlaps the areas most consistently found to be affected in neuropathological investigations of autism brains: the limbic system, cerebellum and cerebral cortex [42], which makes this gene an attractive susceptibility gene for ASDs.

Using BAC array-CGH we identified a 931 kb deletion comprising 7 genes on chromosome 8q22.3. Copy number variations have not been described for this region [191]. Two of the deleted genes namely *BAALC* (Brain and Acute Leukemia Gene, Cytoplasmic) and *RIMS2* (Rab3A-interacting molecule 2) which are both expressed in brain are of special interest with regards to ASDs: *BAALC* is a gene that is absent in lower organisms but is highly conserved amongst mammals [192]. Two protein isoforms

have been detected in brain. In rat, one of these isoforms is located in lipid rafts in the postsynaptic membrane (figure 4) [193]. Since postsynaptic lipid rafts are believed to be involved in membrane trafficking, signal transduction or regulation of the actin cytoskeleton, and since disruption of these rafts leads to depletion of excitatory and inhibitory synapses, loss of dendritic spines and instability of surface AMPA receptors, the importance of these functional entities in normal synapse function is substantiated [193, 194]. Moreover, the developmental protein expression profile of rat BAALC is in parallel with the profile of synaptogenesis in the forebrain and thus suggests a role of BAALC in this process [193].

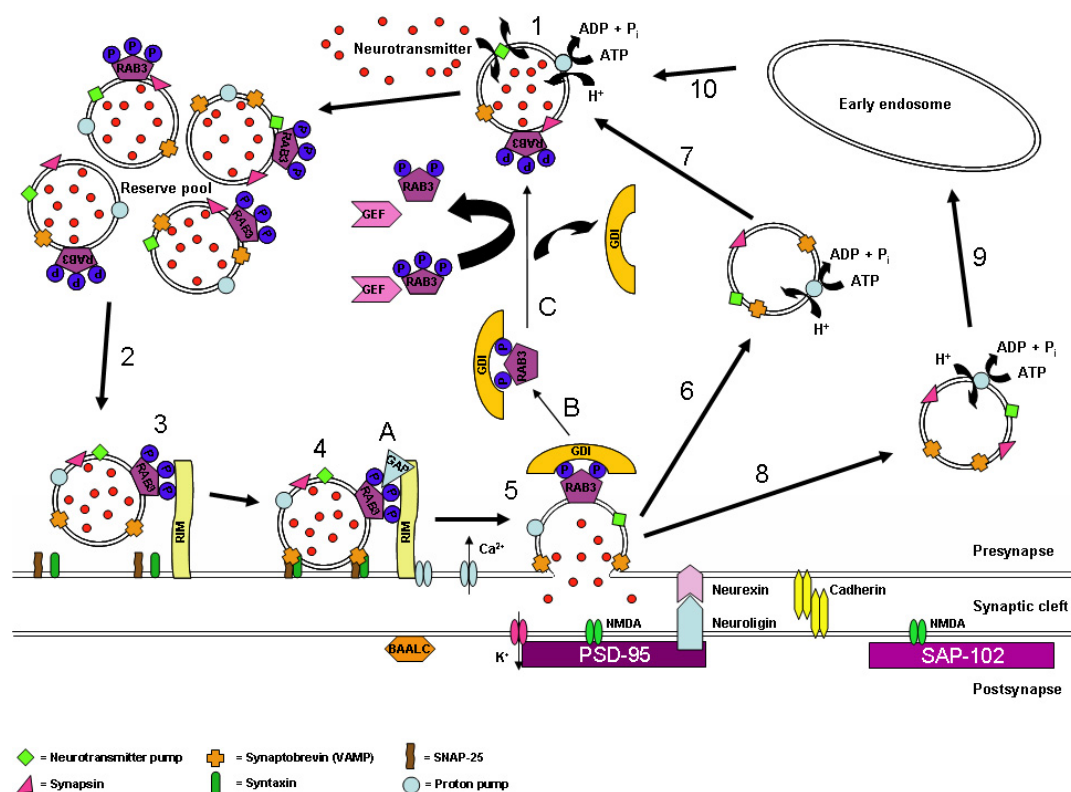


Figure 4. Schematic view of the location and function of synaptic proteins that have been found mutated in patients with ASDs, mental retardation and/or epilepsy. 1) Synaptic vesicles are filled with neurotransmitter by active transport. 2) Vesicles can be recruited to the active zone in the presynaptic membrane. 3) The recognition of RAB3-GTP by RIM proteins is involved in docking and fusion of synaptic vesicles. 4) The SNARE proteins Synaptobrevin, Syntaxin and SNAP-25 promote vesicle priming. 5) Ca^{2+} triggered fusion-pore opening. 5-10) The synaptic vesicles undergo endocytosis and recycle via several routes. A) RAB-GAP accelerates intrinsic GTPase activity of RAB3 [195] B) RAB-GDI prevents dissociation of GDP from RAB3 and solubilizes RAB3 from membranes [195] C) RAB-GEF increases the GDP dissociation rate [195]. Neuroligin and its binding partner, neuroligin, is involved in synapse formation. PSD95, PSD97 and SAP-102 are large MAGUK proteins that couple transmembrane signaltransducers (NMDA receptors and K^+ channels) to intracellular signalling pathways and the cytoskeleton. BAALC is located in lipid rafts at the postsynaptic membrane. This figure is adapted from [196].

The RIM family of proteins includes RIM1, RIM2, NIM1, NIM2 and NIM3 that are all located in the active zone of the presynaptic membranes (figure 4) and probably functions as protein scaffolds that coordinate the docking and fusion of neurotransmitter vesicles [197]. *RIM* genes are almost exclusively expressed in the brain [198]. RIM2 shows high homology to the more thoroughly investigated RIM1 protein [198]. Both RIM proteins exhibit the same overall domain architecture consisting of an N-terminal zinc-finger module, a single central PDZ domain, and two C-terminal C₂ domains [198]. The N-terminal zinc finger domain of RIM proteins binds to the synaptic vesicle protein RAB3 in a GTP-dependent manner whereas the C-terminal C₂ domains associates with the N-type Ca²⁺ channels and synaptotagmin-I (SYT-1) as well as other proteins [197]. Thus, it seems that RIM proteins are RAB3 effectors that assure rapid and efficient synaptic transmission by associating with N-type Ca²⁺ channels and thereby assures docking of secretory vesicles close by [197].

There are no records of a *RIM2* knock out mouse in the literature. However, since *RIM1* and *RIM2* are highly homologous and a knock out mouse of the largest and most abundant isoform of the RIM1 protein, RIM1 α , has been made, it is interesting to speculate whether reduced expression of *RIM2* has a similar effect. RIM1 α , plays an essential role in maintaining normal probability of neurotransmitter release as well as in long-term presynaptic potentiation in the hippocampus and cerebellum and thus is important for associative memory and learning [199, 200]. RIM1 α knock out mice show deficits in maternal behaviour without structural abnormalities in brain architecture [201]. Moreover, increased locomotor activity in response to the mild stress of a novel environment was also observed and interpreted as an abnormal fear response [200]. These observations are interesting in relation to ASDs since deficits in maternal behaviour of RIM1 α ^{-/-} mice might be a sign of abnormal social interaction in the mouse, and this is one of the key diagnostic criteria for ASDs. Moreover, an abnormal stress response to novel situations and environments as seen in the RIM1 α ^{-/-} mice is also a very common observation in autistic patients.

An 874 kb maternal duplication on 5p14.1 comprising exon 1 and 2 of the 12 exons in *CDH9* was identified by BAC array-CGH in the twins. In the database of genomic variants [191] a variation of 179.5 kb overlapping the 3' end of *CDH9* was reported in a normal control subject. However, it is unfortunately not stated whether this region is deleted or duplicated and thus does not help us to infer whether our finding is of

clinical importance. Cadherins are Ca^{2+} dependent adhesion molecules (figure 4) that mediate homophilic binding and are important in tissue histogenesis, neural differentiation, neurite outgrowth and synapse formation [202, 203]. The essential role of cadherins in synapse formation appears to be mediated early in synaptic development since early disruption of cadherin function affects synaptogenesis and subsequent synaptic stability [203, 204]. Intracellularly, cadherins interact with the actin cytoskeleton and several signalling pathways as well as play a role in localizing synaptic vesicles at nascent synapses [204]. In rat, *CDH9* is most extensively expressed in CA3 in hippocampus but expression was also found in the basolateral nucleus of the amygdala, the epithelial layer lining the ventricles and covering the surface of the brain and the cingulate cortex [202]. Since cadherins seem to play an indispensable role in synapse formation it is imaginable that disruption of these genes can be involved in the development of ASDs.

Defective synapse function has previously been identified as an underlying cause of some cases of ASDs and Asperger syndrome where *NLGN3* (neuroligin 3) or *NLGN4* (neuroligin 4) have been mutated [34]. Neuroligins are postsynaptic membrane proteins that are required for proper synapse maturation and brain function [73]. Mutations in *NLGN3* and *NLGN4* have been identified in patients with childhood autism, Asperger syndrome and mental retardation [34, 35]. The mutations identified in autistic individuals impairs cell surface transport of the mutant proteins, but do not completely abolish synapse formation activity [205-207]. Likewise, several genes encoding synaptic proteins are mutated in individuals with mental retardation and epilepsy that are common comorbid diagnosis to ASDs: *DLG3* (*homolog of disc large 3*) encoding SP102, *DLG1* (*homolog of disc large 1*) encoding SAP97, *SYN1* (*synapsin I*), *RabGDI α* (*Rab GDI-dissociation inhibitor α*), *RAB3GAP* (*Rab3 GTPase activating protein*), *MECP2* (*methyl CpG binding protein 2*), *TSC1* (*tuberous sclerosis 1*) encoding hamartin or *TSC2* (*tuberous sclerosis 2*) encoding tuberin [53, 60, 208-215]. In figure 4 the location and functions of these defect synapse proteins are depicted. It is conceivable that mutations in any of the genes encoding proteins involved in the processes depicted in figure 4 may lead to similar phenotypes and thus also suggests that *RIM2*, *BAALC* and *CDH9* as well as other binding partners may be susceptibility genes for ASDs.

In another patient diagnosed with childhood autism we have identified a deletion including *BRUNOL4* and two of the five ultra conserved sequences (UCSs) located close

to this gene (**paper I**). The UCSs are defined as sequences ≥ 200 bp with 100% identity in the human, mouse and rat genome [118, 152]. We sequenced the five UCSs that are located close to *BRUNOL4* in 157 patients with ASDs (including the present twins). We identified a nucleotide change in uc.432 in the twins and their father. This nucleotide change was not found in 167 controls.

BRUNOL4 belongs to the elav (embryonic lethal abnormal visual system) family of RNA-binding proteins that is involved in neuronal differentiation and maintenance [119, 145, 216, 217]. This family of genes is important in posttranscriptional regulation of gene expression such as alternative mRNA splicing, regulation of translation and rate of mRNA turnover [119, 145]. These processes have proved important not only in synthesizing somatic proteins, but also in selective targeting of these proteins to synapses as well as regulated translation of dendritically localized mRNAs near synaptic contacts, which is crucial in inducing synaptic plasticity [217, 218]. Genes encoding proteins with similar functions, e.g. *FMR1* (*fragile X mental retardation 1*), are mutated in several types of X-linked mental retardation [146] which substantiates their possible role in the development of ASDs. In addition, when knocking out the gene encoding the RNA-binding protein translin in mice, a male-specific deficit in learning and memory, locomotor activity, anxiety-related behaviour and sensorimotor gating is noted. This is interesting in concern to ASDs considering the pronounced preponderance of males compared to females [219]. All in all these findings suggest that this group of RNA-binding proteins is important for neuronal development and maintenance in general and synaptic development and function specifically and thus may be susceptibility genes for ASDs.

The most consistently suggested inheritance pattern for ASDs involves transmission of several epistatic genes [2] and thus fits either an oligogenic or polygenic model of inheritance. The existence of a threshold or point of balance has been postulated [156] meaning that a threshold is reached when a sufficient number of susceptibility genes are inherited from both parents. In line with this theory, the mother of our patients is hemizygous for *RIM2*, *BAALC* and *CDH9*, and the father is hemizygous for *ZBTB7C* and has a nucleotide change in an UCS that is probably associated with *BRUNOL4*. It is conceivable that ASD arose in our patients due to the additive effects of these alterations. This does not exclude the involvement of genetic factors that were not detected by us. However, the study suggests that detailed molecular dissection of patients with multiple inherited chromosomal rearrangements may reveal information

about susceptibility genes for ASDs. The common functional theme of the candidate susceptibility genes identified in this study suggests that synaptic dysfunction is the basis for ASD in the Swedish twins presented here. The numerous identified mutations in genes encoding synapse proteins in patients with autism, mental retardation, and epilepsy as described above substantiates that abnormal development of synapse function as well as a skewed balance between neurotransmitter systems may indeed be the underlying cause of ASD in some individuals [41, 43, 220]. Thus, we find it most likely that future genetic research will elucidate changes in the properties of complex gene networks involved in synaptogenesis and/or synapse maintenance in ASD patients.

ACKNOWLEDGEMENTS

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SUPPLEMENTS

Name of sequence	Primer sequence	Product length
ZNF_cDNA_2	5' GGT CGG GCA GCG GGA AGT AGG G 3'	403 bp
	5' TGT GAC CAC CTG CAC CGC CAC A 3'	
ZNF_cDNA_1	5' GTG TGG AAA GAA GTC CGG GGC GAA TG 3'	363 bp
	5' GAC AAG GAG GAC GAT GAC GAC GAC GAA GA 3'	

Supplementary table 1. Primers for southern blot probes.

Name of Sequence	Primer sequences	Length	Melting temp.
Uc429	Forward: 5' GCA CTG CTG GTG TTT AAG 3'	412 bp	57°C
	Reverse: 5' GGC TCT GTA TCT AAA AGT GTG 3'		
Uc430	Forward: 5' CCG TCC CAA CAT ACA CTC AC 3'	519 bp	57/60 °C
	Reverse: 5' AGA GGG GGA GGT TTA GGA TTT 3'		
Uc431	Forward: 5' TGT CAT AAT CAA AAG GCG GGA CTA CAG 3'	480 bp	57 °C
	Reverse: 5' GCT TGC CCA CCA ACT AAC GAG AAC 3'		
Uc432	Forward: 5' GTC TAT CTT CTC ATG TGT CAG CAA CAA GT 3'	529 bp	57 °C
	Reverse: 5' ACT CCT TCC ATT ACC CTC GCT AAA C 3'		
Uc433	Forward: 5' GCA AGC AAT TAC TGG CGA TTT GT 3'	424 bp	57 °C
	Reverse: 5' TGT ATG GTG GCT CGC AAA GG 3'		

Supplementary table 2. Primers for dHPLC screening and sequencing of UCSs.

Name of sequence	Primer sequence	Length
CHR7	Forward: 5' ATA AAG GTT CCT GTG ATG TCA G 3'	266 bp
	Reverse: 5' GTAAGTTATTTAGATGATTTTGAATGC 3'	
CHR13	Forward: 5' ATT AAA CAT GAT AAA AGG GAT TCT TAC 3'	213 bp
	Reverse: 5' ATA ATG AGC ACT TAG AGC AGG A 3'	
CHR20	Forward: 5' AAA CGT GTG CTC TTT TCC CCA TCT TAT 3'	354 bp
	Reverse: 5' GCT AGC CCT CTC TGG ATT CCT TCT TC 3'	

Supplementary table 3. Primers used for PCR check of genomic contamination of cDNA

7 PAPER V

Breakpoint Cloning and Haplotype Analysis Indicate a Single Origin of the Common Inv(10)(p11.2q21.2) Among Northern Europeans

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ABSTRACT

The pericentric inv(10)(p11.2q21.2) has been frequently identified in cytogenetic laboratories, is phenotypically silent and is considered to be a polymorphic variant. Cloning and sequencing of the junction fragments on 10p11 and 10q21 revealed that neither inversion breakpoint directly involved any genes or repetitive sequences, although both breakpoint regions contain a number of repeats. In our series of apparently unrelated inv(10) families, all 20 had identical breakpoints and detailed haplotype analysis showed that the inversions were identical-by-decent. Thus although considered a common variant, the inv(10)(p11.2q21.2) has a single ancestral founder among Northern Europeans.

A small number of cytogenetically visible human chromosome rearrangements are considered to be polymorphic variants, including several common pericentric inversions [221]. These inversions fall into two classes: one where both breakpoints occur within heterochromatin (chromosomes 1, 3, 9 and 16) and the other where both breakpoints occur within euchromatin (chromosomes 2, 5 and 10). The heterochromatic variants are the most frequent but may be a consequence of alterations in the amount and distribution of heterochromatin rather than true inversions.

The pericentric inv(10)(p11.2q21.2) is not associated with any phenotypic abnormalities [222] and has been frequently identified in cytogenetic laboratories in the UK [222], France [223], Denmark and Sweden [224], and North America [225]. The estimated frequency of the inv(10) among prenatal diagnostic referrals to the laboratories taking part in this study is 1 in 3600 in Germany, 1 in 7100 in Denmark and 1 in 12800 in the UK. Thus while the great majority of chromosome inversions appear to be unique rearrangements, the frequency and wide geographical distribution of the inv(10)(p11.2q21.2), suggests that it might be a recurrent variation that has arisen independently in different populations [226].

Repetitive sequence elements have been implicated in the formation of a range of recurrent structural rearrangements [227]. For example, the breakpoints of the most frequently occurring non-Robertsonian translocation, t(11;22), are within palindromic AT-rich repeat sequences (PATRRs) [228] and low copy number repeats (LCRs) or duplicons mediate the formation of microdeletions and microduplications [229].

No	Patient	Country of Origin
1	Ger1	Germany
2	Sw1	Sweden
3	Sw2	Sweden
4	Dk1	Denmark
5	Dk2	Denmark
6	Dk3	Denmark
7	Uk1	United Kingdom
8	Uk3	United Kingdom
9	Uk4	United Kingdom
10	Uk5	United Kingdom
11	Uk6	United Kingdom
12	Uk7	United Kingdom
13	Uk8	United Kingdom
14	UK9	United Kingdom
15	UK10	United Kingdom
16	Ger2	Germany
17	Ger3	Germany
18	Ger4	Germany
19	Ger5	Germany
20	Rus1	North Western Russia

Table 1. Study Population. Bold = phase known

We have studied a series of 20 apparently unrelated families with cytogenetically identical inv(10)s comprising nine families from the UK, five from Germany, three from Denmark, two from Sweden and one from North Western Russia (Table 1). Our study had two specific aims: (1) to characterize the inv(10) breakpoints at the molecular level to ascertain whether the formation of the inversion is mediated by repetitive sequence elements and (2) haplotype analysis to determine the proportion of inv(10)s which arose independently and the proportion which share an ancestral founder and are identical-by-descent (IBD).

The inv(10) breakpoints of patients 1 and 2 were located by fluorescent *in situ* hybridization (FISH) in the cytogenetic bands 10p11.21 and 10q21.1. For both inv(10) carriers the BAC clone RP11-92B19 spans the breakpoint on 10p11.21. On 10q21.1 the breakpoints of both carriers were within the overlapping region of BAC clones RP11-

22H3 and RP11-806B6. Subsequent analysis showed that the breakpoints of a further seven inv(10) carriers fell in the same spanning BAC clones [230].

A)

10pter - GTAGTAATGTATGCATTTGTAATAGTAATAGTTAACATTACCA

GTAGTAATGTATGCATTTGT–**TAGTAATAGTTAACATTACCA**

ATCAATTAGTGTATTCATAATTCTATGTAATGTAATTATTATTAA - 10qter

ATCAATTAGTGTATTCATAA–CTATGTAATGTAATTATTATTAA

B)

Sequence of the 10p	GTA	TGC	ATT	TGT	AA	<div style="border: 1px dashed black; padding: 2px;">TTA</div>	TGA	ATA	CAC
junction fragment	CAT	AGC	TAA	ACA	<u>TT</u>	<div style="border: 1px dashed black; padding: 2px;">AAT</div>	<u>ACT</u>	<u>TAT</u>	<u>GTG</u>
Sequence of 10q	ACA	ATT	ACA	TAG	TAG	TAA	TAG	TTA	
junction fragment	<u>TGT</u>	<u>TAA</u>	<u>TGT</u>	<u>ATC</u>	ATC	ATT	ATC	AAT	

Figure 1. Sequence Analysis of Junction Fragments. (A) Genomic sequence encompassing breakpoints. (B) Sequence of PCR-amplified junction fragments showing the chromosome 10 genomic sequence (normal text), the sequence flanking the 10p11 breakpoint (white text), the sequence flanking the 10q21 breakpoint (underlined text), and the sequence from either 10p11 or 10q21 (boxed). The genomic sequences shown have the following coordinates in NCBI 35 (November 2005): 10p: 37,148,066 – 37,148,108 / AL390061.9; 4,483 – 4,525 and 10q: 59,748,173 – 59,748,217 / AC016396.6; 120,582 – 120,626.

The inversion breakpoints of patient 1 were further refined by Southern blot analysis and subsequently cloned. Sequence analysis revealed an overall loss of two nucleotides. The break in the 10q junction fragment could be unambiguously assigned, but in the 10p junction fragment there was a 2 bp overlap common to both 10p11 and 10q21 sequences (Figure 1). Thus it is not possible to tell at which breakpoint site the deletion occurred. Apart from the 2 bp identity at the breakpoint, there was no extensive homology between the 10p11 and 10q21 sequences.

To determine whether other inv(10) carriers in our series contained the same breakpoints, we designed PCR assays to specifically amplify the rearranged chromosome 10 (Table 2). PCR fragments of identical length were amplified at both breakpoints in the remaining 19 families and sequencing revealed that the breakpoints were identical in all the inv(10) carriers.

The 10p11 breakpoint maps to 37,148 kb from 10pter (NCBI 35, Nov 2005) in a gene desert with no known gene for 300 kb on either side of the breakpoint. The 10q21 breakpoint maps to 59,748 kb within a cluster of four genes (*IPMK*, *CJ070*, *UBE2D1* [MIM 602961] and *TFAM* [MIM 600438]). Although a position effect cannot be excluded, no genes are directly disrupted by either breakpoint. This observation is consistent with the benign nature of the inversion.

Breakpoint	Primer Sequences	Fragment Size	PCR Conditions
10p11.2	GAGGCCAGGCTTAAA GCAACCCCACTATGGTCTGCACCAG	354 bp	39 cycles: 95°C 30s, 57°C 30s, 72°C 40s
10q21.2	AGCTGCTGTAGCCTTTGCAC AACTGGTAAAAGAAGATCCTTGG	513 bp	39 cycles: 95°C 30s, 56°C 30s, 68°C 40s

Table 2. Amplification of 10p11 and 10q21 Junction Fragments.

The breakpoints did not directly involve any repetitive sequences. However, although the breaks occurred within short stretches of unique single copy sequence, in both cases these were flanked by several repeats. The RepeatMasker program showed that the sequence around both breakpoints was enriched for interspersed repetitive elements. The 10 kb interval on 10p11, 5 kb on either side of the breakpoint, contained 34% repetitive sequences (15% SINES, 14% LTRs) and the 10 kb interval on 10q21 contained 47% repetitive sequences (20% LINEs, 10% LTRs, 9% SINES). Interspersed repeats may promote instability and the formation of DNA double strand breaks and/or act as substrates for recombination [227]. Therefore, while it seems unlikely that the sequences around each breakpoint predisposed to the formation of the inversion, we cannot exclude this possibility.

The presence of the same breakpoints in all inv(10) carriers and the lack of obvious predisposing factors suggest a founder effect, i.e. that all 20 families share a common ancestor. In order to determine whether the inv(10)s were all IBD we undertook detailed haplotype analysis using microsatellites and SNPs. DNA was available for more than one inversion carrier from five of the 20 families. The five haplotypes for which phase was known were identical or differed at no more than two of the 17 microsatellites tested within the inversion, (Table 3). This suggests that all five inv(10)s are IBD and allowed us to predict the likely ancestral haplotype which was identical to that observed

for family 8 (UK 3). In contrast to the degree of allele sharing within the inverted region, the flanking haplotypes were completely divergent outside the inversion breakpoints.

We also typed the same microsatellites in the 15 families where DNA was available from only a single carrier (Table 4). This demonstrated that all 20 families are IBD. The alleles in eight of the 20 families were consistent with the common haplotype, while in 12 families there was at least one difference. In total there were nine allele differences: five were private mutations, while four were seen in more than one family. The most common allele change observed was at the microsatellite D10S220 from a PCR product length of 107 bp in the ancestral haplotype to 109 bp in five families.

For SNP analysis (Supplementary Table 1) we compared three families where phase was known and four families where phase was unknown. In contrast to the microsatellites, which spanned the whole inversion, SNPs were chosen in the immediate vicinity of the breakpoints over a few kb. All seven inv(10) families tested had exactly the same haplotype (Table 5) providing further evidence that the inversions are IBD. Families 2 (Sw1) and 3 (Sw2) had identical haplotypes even though they differed at four of the 17 microsatellites. This is likely to be due to the higher mutation rates in microsatellites compared to SNPs. Thirty-six control SNP haplotypes were generated from 18 normal individuals (from nine trios) to assess the frequency of the inversion haplotype. There were 19 different haplotypes and in total six of the 36 control chromosomes carried the inversion haplotype (one homozygous and four heterozygous individuals). Thus it is unlikely that the SNP haplotype shared by the inv(10) carriers is coincidental.

The haplotype analysis demonstrated complete suppression of recombination within the inverted segment. Our data can not distinguish between a direct effect, i.e. crossing over does not occur, or indirect selection against unbalanced recombinant products. The inversion breakpoints are close to the centromeric areas of low recombination. No recombinants were seen in two studies of 33 and 15 inv(10) families [222, 224].

Het	Alleles	Mb	Locus	Family 2	Family 3	Family 8	Family 9	Family 15	Founder
0.84	10	28.7	D10S600	178	182	190	186	182	-
0.83	9	29.5	D10S213	188	180 . 188	188	172	182	-
0.76	12	29.7	D10S204	291	291	319	329	295	-
0.82	9	30.6	D10S193	214	220	224	224	220	-
0.80	9	31.7	D10S208	180	182	182	178	182	-
0.86	12	32.4	D10S199	173	173	173	179	179	-
0.72	10	33.7	D10S1666	266	258	276	274	256	-
1.00	unknown	33.9	D10S1175	320	320	316	310	348	-
0.70	10	36.8	D10S176	114	94	94	94	94	-
0.73	6	37.1	D10S1791	207	201	201 . 207	201	201	-
		37.1	10p11.21						
0.67	unknown	37.8	D10S508	184	184	184	184	184	184
		Centromere 39 – 41							
0.85	13	42.8	D10S141	115	115	115 . 131	115	115	115
0.87	10	42.8	D10S469	123	123	123 . 137	123	123	123
0.84	10	44.8	ZNF22	151	151	151	151	151	151
0.90	17	48.0	sJRH	303	299	299	299	299	299
0.86	11	49.5	D10S1793	254	254	254	252	254	254
0.75	5	50.4	D10S1766	171	171	171	171	171	171
0.84	10	51.7	D10S220	107	107	107	107	109	107
0.79	6	51.8	D10S196	100	94	100	100	94 . 100	100
0.84	11	54.6	D10S1790	191	193	193	193	193	193
0.76	8	54.7	D10S539	93	93	93	93	93	93
0.88	14	56.8	D10S1124	231	231	231	231	231	231
0.78	7	57.3	D10S1788	249	249	249	249	249	249
0.71	13	58.0	D10S1767	256	256	256	256	256	256
0.84	9	58.4	D10S1756	192	192 . 194	192	192	192	192
0.88	unknown	58.6	D10S524	369	365	369	369	369	369
0.75	8	58.7	D10S1659	184	184	184	184	184 . 194	184
		59.8	10q21.1						
0.79	8	60.8	D10S589	190	186	184	184	186	-
0.78	8	60.9	D10S464	140	140	144	134	140	-
0.78	10	63.8	D10S1652	167	163	165	161	171	-
0.80	12	65.2	D10S581	142	134 . 138	136	136	148	-
0.78	9	66.8	D10S1743	227	-	235	243	241	-
0.76	12	68.2	D10S1670	305	301	305	305 . 307	321	-
0.80	6	69.4	D10S210	135	-	133	129 . 133	131	-
0.82	9	70.3	D10S1647	204	208	212	208	206	-
0.87	12	70.6	D10S1665	240	-	238	218	234 . 240	-
0.83	9	71.7	D10S537	-	-	298	290	292	-
0.85	12	72.6	D10S1650	136	-	132	124	138	-

Table 3. Microsatellite Analysis in Families where Phase is Known. The first four columns give details of the microsatellites used, the next five columns show the allele sizes for each family and the last column shows the inferred ancestral haplotype. Allele sizes are taken from the total size of the PCR product and are given in base pairs to the nearest whole number. Breakpoints are shown as horizontal black bars and the centromere as a grey bar. All microsatellite details are available from the Genome Database and distances were taken from Ensembl. Alleles outside the inversion are in *italics*. Shared alleles and the common haplotype are shown in **bold**, allele differences are highlighted in white text.

Locus	Ancestral Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
10p11.21																					
D10S508	184	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Centromere																					
D10S141	115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S469	123	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ZNF22	151	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sJRH	299	-	303	-	-	-	-	-	-	-	-	-	-	-	-	-	-	303	303	-	-
D10S1793	254	-	-	-	-	-	-	-	-	252	-	-	-	-	-	-	-	-	-	-	-
D10S1766	171	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S220	107	109	-	-	-	-	-	-	-	-	109	109	-	-	-	109	-	-	-	-	109
D10S196	100	-	-	94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S1790	193	-	191	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S539	93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S1124	231	-	-	-	-	-	-	-	-	-	-	213/223	-	-	-	-	-	-	-	-	-
D10S1788	249	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S1767	256	-	-	-	-	-	-	-	-	-	-	-	254	254	-	-	-	-	-	-	-
D10S1756	192	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	190	190	-	-
D10S524	369	-	-	365	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D10S1659	184	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10q21.1																					

Table 4. Microsatellite Results for All 20 Inv(10) Families. The common ancestral haplotype is shown in the second column. Subsequent column show the results for each family. A dash “-” indicates the same allele is present as in the ancestral haplotype. Where no allele is shared with the ancestral haplotype, i.e. a microsatellite mutation, the size of the divergent allele is shown .. Allele sizes are taken from the total size of the PCR product and are given in base pairs to the nearest whole number Only microsatellites within the inverted region are shown.

It is difficult to make an accurate estimation of the age of the inversion. The geographical distribution of the 20 inversion carriers, the accumulation of microsatellite mutations within the inversion, estimates for which range from 10^{-2} to 10^{-4} per locus per generation, and the occurrence of crossovers very close to both the 10p11 and 10q21 breakpoints in most, if not all, families suggest that the rearrangement is not a recent event. This is consistent with the calculation of average reproductive fitness for inversions of 0.926 ± 0.085 [231].

The breakpoints of a small number of other pericentric inversions have also been determined. In contrast to the inv(10), these inversions were studied because they were associated with specific abnormal phenotypes and consequently the majority of breakpoints were identified within the introns of genes [232-236]. Graw et al. [237] cloned the breakpoints of the inv(8)(p23.1q22.1) which is associated with various clinical manifestations including mental retardation and heart defects in unbalanced carriers (Rec 8 syndrome, MIM 179613). The results were similar to the inv(10) in a number of ways: no genes were directly disrupted by the inversion; the breakpoint sequences showed little homology; the breakpoints lay in unique sequences flanked by repetitive elements and the inversion has spread widely from a single founder.

The 20 inv(10) families studied were all from Northern Europe. It would be interesting to establish whether all cases worldwide are also derived from the same founder. Of the inv(10) cases in the literature only one has been reported as *de novo* [238]. Breakpoint sequencing and haplotype analysis should be applied to any potentially unrelated or non-European inv(10) carriers. We have contacted several cytogenetic laboratories worldwide whose populations are unlikely to be of European origin. To date we have had replies from three laboratories (in Egypt, Mexico and Singapore), none of whom have identified a single inv(10). The only non-European cases in the literature are from the USA and Canada [223], and these could conceivably be individuals of European origin.

Thus the overall evidence suggests that although it is considered a common variant, the inv(10) may well be a unique rather than a recurrent rearrangement, with a single European founder. It would be interesting to apply the approaches used in this study to other common inversions, such as the variant inv(2)(p11q13), to establish whether they are also identical-by-descent.

10p	Alleles	Inv(10)
rs3898062	A / G	G
rs2488748	C / G	G
rs12257945	A / G	A
rs12572077	A / C	A
rs2695081	C / G	G
rs4934835	C / G	C
rs11010897	A / G	A
Breakpoint		
rs2463226	A / T	A
rs10827731	C / T	C
rs11817755	G / T	T
rs3867222	C / T	T
rs2490841	G / T	T
10q	Alleles	Inv(10)
rs16851	C / T	T
rs11818916	A / C	A
rs2486489	G / T	T
rs12248484	A / G	G
Breakpoint		
rs7072568	A / G	G
rs12241885	C / T	C
rs1007915	C / T	T

Table 5. Conserved SNP Haplotype. Twelve SNPs around the 10p breakpoint and seven SNPs around the 10q breakpoint were selected for SNP analysis by enzyme digestion or sequencing. All details are given in Supplementary Table 1.

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WEB RESOURCES

Ensembl	http://www.ensembl.org/
Genome database	http://www.gdb.org/
Repeat Masker	http://www.repeatmasker.org/
Online Mendelian Inheritance in Man	http://www.ncbi.nlm.nih.gov/omim

SUPPLEMENTS

Chr.	SNP	Sekv/ enz.	SNP	Primers	PCR fragment	PCR conditions	Restriction enzymes	Restriction fragments
10p	rs3898062	Enz.	A/G	5' AGGAGAATGGCGTGAATC 3' 5' GATATTATCATGGAGGCTTTAGG 3'	344 bp	Platinum taq. 96° C 5 min.; (96°C 30s.; 59.8-66.4°C 30 s.; 68°C 30s)x39; 68°C 10 min.	Mse I	A: 29, 42, 117, 156 bp G: 42, 146, 156 bp
	rs2488748	Enz.	C/G	5' GTGGCAAGAAAGCTAGTAAGT 3' 5' TCAAATAGCAGAGTCGCA 3'	320 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Fnu4HI	C: 81, 239 bp G: 58, 81, 181 bp
	rs12257945	Sekv.	A/G	5' TTGTATTAGAGGAACCCGAAGCA 3' 5' GGAGCCAGTGTAAACACGGTAGAT 3'	324 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs12572077 .rs2695081	Sekv.	A/CG/C	5' CATCTTTTCCCCACCATAGTGT 3' 5' CTCAGCCACAGATTCAAGTTCC 3'	272 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs4934835	Sekv.	G/C	5' aggctgctcaataaacacgtgaa 3' 5' GTGGTGGTGTACCCGTGGTC 3'	224 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-68°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs11010897	Enz.	A/G	5' GTACACCACCACAGCTAATG 3' 5' GTTGGCTATAATACGGAGTTCTA 3'	354 bp	Platinum taq. 96° C 5 min.; (96°C 30s.; 48-59.8°C 30 s.; 68°C 30s)x39; 68°C 10 min.	Alu I	A: 14, 108, 232 bp G: 14, 340 bp
	Breakpoint							
	rs2463226	Sekv.	A/T	5' CAAGGATCTTCTTTTACCAGTTA 3' 5' AATGAACTAATTTTATCATAGGCT 3'	155 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs10827731	Sekv.	C/T	5' GCACCAATATAAAAATGACTCAAA 3' 5' CTCAGAGCAGCCAGAGTG 3'	170 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs11817755	Enz.	G/T	5' GAGGTGTGGAAGGAGAGGCGCAGGT 3' 5' AGCTGAGGCCCGGCAAGAATTTGAGT 3'	239 bp	Platinum taq. 96° C 5 min.; (96°C 30s.; 69-70°C 30 s.; 68°C 30s)x39; 68°C 10 min.	Alu I	G: 2, 20, 74, 143 bp T: 2, 20, 60, 74, 83 bp
	rs3867222	Enz.	C/T	5' GTTCCATCACAGGTCATCTT 3' 5' CAATAAAATGTTGGGCTATTAAA 3'	332 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 48-59.8°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Tsp45I	C: 332 bp T: 83, 249 bp
	rs2490841	Enz.	G/T	5' TGGCTGTGTTTTTTCAGATTGG 3' 5' GAGAGGGAAGAGCAATGACA 3'	272 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 48-63.7°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Alu I	G: 78, 194 bp T: 272 bp
10q	rs16851	Enz.	C/T	5' TATCTACACATTCATTATTCCC 3' 5' CATAATATATGTCAGGCGTTTG 3'	195 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 48-56.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Taq I	C:44, 151 bp R:195 bp
	rs11818916	Enz.	A/C	5' TGGATGTTTGATGGAGTTGGTAGTTTTG 3' 5' GTAGTGTCTGCTGGGTTCAACCGAA 3'	199 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 48-68°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Tsp45I	A: 61, 138 bp C: 199 bp
	rs2486489	Sekv.	G/T	5' TTCCCCCACAACATCTCAACTG 3' 5' TGGTTTCCAGCTAGTAGATTTGAATCCAC 3'	221 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-66.4°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs12248484	Sekv.	A/G	5' AGTCTGATTGTGGCTATTTCG 3' 5' TATACTGTTAGCCTCTGACCCAT 3'	252 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 51.2-63.7°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs4644605	Sekv.	C/T	5' ATTTTGGGGGATAGGAGTATGTG 3' 5' TTTAGCATTTGGGCAGTCATAA 3'	271 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-59.8°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	Breakpoint							
	rs7072568	Sekv.	A/G	5' AGGTGGGCGGGATGTTAATGT 3' 5' TGACCGGGAGAAAAGGCTTAAGA 3'	272 bp	Ordinary Taq. 96° C 5 min.; (96°C 30s.; 48-64.8°C 30 s.; 72°C 30s)x39; 72°C 10 min.		
	rs12241885	Enz.	C/T	5' CATAAATGCCCCGATTGCCGACT 3' 5' AGGGATCTTGCGAGCCGTCAGAA 3'	307 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 64.8-68°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Fnu4HI	C: 12, 50, 61, 184 T: 12, 50, 245 bp
	rs1007915	Enz.	C/T	5' TACACTTCCTTCCTCCTGCGTAG 3' 5' AGATGTGGGCACCAGGATATG 3'	334 bp	Ordinary taq. 96° C 5 min.; (96°C 30s.; 56.4-64.8°C 30 s.; 72°C 30s)x39; 72°C 10 min.	Mse I	C: 81, 116, 137 bp T: 12, 81, 116, 125 bp

Supplementary table 1. Primers and PCR conditions used to amplify PCR product for SNP analysis. Restriction enzymes as well as size of restriction fragments are stated.

8 DISCUSSION

The phenotypical spectrum of ASDs displays considerable variation even when contemplating only the clinically best defined diagnosis “childhood autism”. Some individuals have severely mental retardation whereas others have a non-verbal IQ above average; some individuals obtain verbal fluency whereas others remain mute; some individuals have comorbid epilepsy, phobia, tourette syndrome, OCD, ADHD, major depression, bipolar disorder or schizophrenia whereas others do not [3, 14, 16, 17, 20, 185, 186]. When including the phenotypes observed in close family members the variation is even greater and involves delayed onset of speech, difficulty with reading, bipolar disorder, major depression, schizophrenia and anxiety disorders in addition to the broader autism phenotype [2, 10, 12-15]. It is therefore necessary to consider different possible explanations for this variation.

A poly- or multigenic inheritance pattern of several epistatic genes is the most consistently suggested explanation for the observed phenotypical variation [2]. According to this theory both parents contribute with one or more susceptibility genes that in conjunction result in the ASD phenotype but separately or in a different genetic setting have different phenotypical outputs that might or might not be within what is considered the normal spectrum [2]. Hence, the variation in ASD phenotype is an outcome of the number and nature of the inherited susceptibility genes. **Paper II** and **paper IV** support this inheritance model. In **paper II** *KCNQ3* is disrupted by a *de novo* translocation in a, male patient with childhood autism. Moreover, there might be additional susceptibility genes for ASDs on both the maternal and paternal side of the family since the mother has seasonal affective disorder, the mother’s sister has a son with behavioural difficulties and a paternal cousin has a son diagnosed with Asperger’s syndrome. Hence, we have suggested that the *de novo* translocation in conjunction with unknown genetic factors inherited from both parents have resulted in childhood autism in this patient. In **paper IV** the monozygotic, male twin couple with ASD has inherited chromosomal rearrangements from both parents. Accordingly, the twins are hemizygous for *ZBTB7C* due to a paternally inherited translocation as well as hemizygous for *BAALC* and *RIM2* because of a maternally inherited deletion and they are functionally hemizygous for *CDH9* due to a maternally inherited duplication. Thus, we suggested that ASD has arisen in the twins due to the additive effects of these (and potentially additional, uncharacterized) genetic

alterations. The parents do not have any psychiatric disorders or abnormal cognitive development.

A different, although related, explanation for the observed variation in phenotype is that different genes are responsible for different diagnostic entities in ASDs (e.g. language impairment, social deficits). This is still a poly- or multigenic inheritance model but the genes do not need not be epistatic or function in the same biological pathways. This inheritance model is suggested in **paper III** where we present a Danish man with ASD and a maternally inherited translocation. The patient is diagnosed with ASD because he only fulfils two of the three core diagnostic criteria for childhood autism: social reticence and impairment in verbal and nonverbal communication. The mother has four children with four different men but only the youngest child lives with the mother. She has supervised custody of this child and has only occasional contact with the proband. Even though the mother does not have a clinical diagnosis and therefore in principle is considered normal the family history support that she has reduced social capacity. The father can not read or write and has impairment of speech. He is most likely either severely dyslexic or mentally retarded. He has two children (younger than the proband) with another woman. Both of these children have attended a day care centre and a school for children with special needs where they have received remedial teaching. Hence, our hypothesis is that the proband inherited the reduced social capacity from the mother and the communication deficit from the father, which adds up to the ASD phenotype.

The third proposed explanation for the phenotypic variation implies that environmental factors can modulate the phenotypical output of identical genetic backgrounds. This effect might also in part be involved in the development of ASD in the proband in **paper III** described above. The maternally inherited translocation disrupts *NUDT6* that presumably regulates *FGF2*. Since *FGF2* is neuroprotective it is possible that altered expression of *FGF2* can increase the vulnerability of selected neuronal populations which in combination with e.g. stress might result in neuronal death.

The fourth possible explanation of the observed phenotype variation is that a major locus is involved in the development of ASD and the variability in phenotype arises either because of different effects of different mutations in the same gene, or due to stochastic variations arising because “genetic programming is probabilistic rather than deterministic” [239]. There are several examples of this for ASDs since mutations in *ARX*

can cause autism, infantile spasms, epilepsy, mental retardation or cerebral malformations [36]; mutations in *NLGN3* and *NLGN4* can cause childhood autism, Asperger's syndrome and mental retardation [34, 35], mutations in *MECP2* can cause childhood autism, Rett syndrome, mental retardation, Angelman syndrome and progressive spasticity [54, 147, 240], mutations in *FMR1* can cause mental retardation and ASDs [57] and mutations in *TSC1* and *TSC2* can cause tuberous sclerosis, ASDs, lymphangioleiomyomatosis, focal cortical dysplasia of Taylor [241, 242].

The last, and in my opinion most likely, explanation for the phenotypical variation is that the ASD spectrum is composed of aetiologically distinct subgroups that overlap in phenotypical spectrum. Therefore, no single model will be valid to all ASD cases but rather all of the above mentioned inheritance models. This is substantiated by the identification of an ASD phenotype in several syndromes and medical conditions with known genetic aetiology: Smith-Lemli-Opitz syndrome (*DHCR7*) [243], Bannayan-Riley-Ruvalcaba syndrome (*PTEN*) [244], Rett syndrome (*MECP2*) [54], Neurofibromatosis (*NF1*) [57], Tuberous sclerosis (*TSC1/TSC2*) [57], Angelman syndrome/ Prader Willi syndrome (15q11-13 copy number variations) [57, 67] among others. Accordingly, no genes will probably be necessary or sufficient to develop ASDs, which poses a problem when looking for susceptibility genes by linkage analysis. This might, however, explain the few reproducible linkage results obtained for ASDs and it substantiates, that it may be difficult if not impossible to identify genetically homogeneous subgroups of ASD patients by their phenotype. In addition, it suggests that alternative methods to identify putative ASD susceptibility genes must be considered.

Defining endo-phenotypes in ASD patients such as "age of first word" is a well known way of trying to unify the genetic aetiology [245]. One could argue that classifying ASD patients according to biologically founded parameters such as "decreased volume of hippocampus as identified by MRI scans" or "increased plasma serotonin" might be more likely to identify genetically homogeneous groups as these are measurable values that are not subject to subjective evaluation.

Approximately 75% of individuals diagnosed with childhood autism have comorbid mental retardation [3]. In addition, mutations in several genes have already been shown to result in both mental retardation and/or ASDs (*MECP2* [54], *NF1* [57, 65], *TSC1* [57], *TSC2* [57], *NLGN3* [34, 35], *NLGN4* [34, 35], *ARX* [36], *AUTS2* [84]) and it might thus be reasonable to suggest that these two disorders for a large part share

genetic aetiology. Accordingly, the numerous mental retardation genes identified on the X-chromosome might be interesting to consider for ASDs as these might in part explain the male preponderance observed in ASDs. Similarly, there might be an overlap in susceptibility genes for ASDs and other psychiatric disorders since they tend to segregate in the same families as ASDs, show considerable overlap in linkage intervals and 72% of patients with ASDs have comorbid other psychiatric disorders [10, 13, 20, 185, 186]. A different approach to identify susceptibility genes for ASDs could therefore be to perform linkage studies in families where the broader autism phenotype or the above mentioned cognitive and/or psychiatric disorders are prevalent or to include these family members in genome wide screens.

The easiest way to circumvent the above mentioned problems is perhaps to characterize observed chromosomal rearrangements at the molecular level and in addition identify additional **copy number variations (CNV)** by array-CGH. This combined strategy makes no assumptions on inheritance models or on whether or not susceptibility genes are shared by other ASD patients. This method has primarily been used to characterize *de novo* translocations in ASD patients from the notion that these chromosomal rearrangements would pinpoint ASD forms caused by a “major locus” mutation. Our results clearly suggest that the strategy is not only suitable for identifying major locus susceptibility genes caused by *de novo* rearrangements but might also pinpoint one or more susceptibility genes for polygenic forms of ASDs caused by familial chromosomal rearrangements (**papers I, II, III, IV**).

However, this method will naturally also identify chromosomal rearrangements, CNVs and genes that turn out not to confer susceptibility to ASDs. As an example we demonstrate in **paper V** that the inv(10)(p11.2q21.2) observed in the Swedish, male monozygotic twin couple described in **paper IV** is identical to the inversions observed in 19 unrelated individuals without ASDs. Hence, this inversion is considered a variant chromosome with no apparent phenotypical implications. This is further substantiated by another study describing 33 families with apparently similar inversions but no overlapping clinical diagnosis [222].

Distinguishing true susceptibility factors from irrelevant genetic variations is a general problem for complex disorders since no single gene is necessary or sufficient to cause the disorder and therefore even true susceptibility alleles will be found in some controls and be absent from some patients, which is in contrast to Mendelian disorders

[44]. Accordingly, looking for mutations in putative susceptibility genes for ASDs in a cohort of patients can not alone reveal the disease-causing potential of the genes but must rather be combined with additional information. Functional studies and association studies are two approaches to gain information about the relevance of suggested susceptibility genes. Another approach could be to pool data of all putative susceptibility genes for ASDs and try to identify common developmental pathways like “neuronal migration”, “synapse function”, “neurite outgrowth” and eventually even more detailed signalling pathways. This would potentially group the disorders into genetically meaningful entities and it would provide a functional framework) for identification of additional putative susceptibility factors (e.g. protein binding partners. Obviously, this is not an easy task since all cellular processes are somehow connected and some proteins are therefore involved in several of the above mentioned pathways, but it might nevertheless reveal that some pathways are more often involved in the aetiology of ASDs than others and thereby sort out relevant from irrelevant genes. In line with this theory approximately 40% of the genes in table 1 (5 of the genes identified by us: *FGF2*, *CDH9*, *RIM2*, *BAALC*, *KCNQ3*) are suggested to encode proteins involved in “synapse function”, which means “synaptogenesis”, “neurotransmission”, “**long term potentiation (LTP)** learning and memory”, and “neurotransmitter production/degradation”. In addition, abnormal synapse function has emerged as an underlying cause of several cases of mental retardation and epilepsy as described in **paper IV**. This further strengthens the aetiological overlap in these disorders and supports the importance of synapse dysfunction in the aetiology of ASDs. In line with this theory, several recent papers review how the normal processes underlying memory formation are changed in Rett syndrome, Neurofibromatosis and Angelmann syndrome that are all frequently associated with ASDs as well as in some mental retardation syndromes [66, 246]. Future experiments will presumably reveal whether abnormal synapse function is indeed one of the main underlying causes of ASDs and furthermore reveal whether the candidate ASD susceptibility genes identified by us are actually involved in disease development.

9 CONCLUSIONS AND PERSPECTIVES

The studies included in this report support the multigenic inheritance patterns for ASDs that have previously been suggested (**paper I, II, III, IV**). Moreover, the candidate susceptibility genes identified in **paper III** (*NUDT6/FGF2*) provide a potential link between a genetic predisposition and an environmental factor (stress) that in a mouse model system result in a male specific effect. Accordingly, *NUDT6/FGF2* are to my knowledge the first autosomal candidates for the male preponderance in ASDs. Thus, in concert with previous findings our studies imply that ASDs are composed of many aetiologically different disorders that may be caused by different types of mutations. As a direct consequence we suggest that characterization of cytogenetically visible chromosomal rearrangements in combination with array-CGH may be a powerful strategy to identify putative susceptibility genes for ASDs. In this regard we propose that not only *de novo* chromosomal rearrangements (**paper I and II**) or rearrangements that segregate with a phenotype in a family are useful but also multiple chromosomal rearrangements inherited from two clinically normal parents might reveal putative susceptibility genes for ASDs (**paper IV**). The candidate susceptibility genes identified include: *BRUNOL4*, *MAPPRE2*, *ZNF397*, *ZNF396*, *ZNF271*, *ZNF24*, *STATIP1*, *GALNT1*, *SLC39A6*, *FAT* (**paper I**); *KCNQ3* (**paper II**); *FGF2*, *NUDT6* (**paper III**); *RIM2*, *BAALC*, *CDH9*, *ZBTB7C* (**paper IV**). In addition, we identified three nucleotide changes in two UCSs that are believed to regulate nearby genes (**paper I**). Moreover, we show that the *inv(10)(p11.2q21.2)*mat identified in a monozygotic twin couple with ASD most likely does not have phenotypical implications (**paper V**).

On the basis of the present (**paper II, III, IV**) and previous findings we suggest that abnormal synapse function (synaptogenesis, LTP/memory and learning, neurotransmission, neurotransmitter formation/degradation) might be the underlying cause of a considerable proportion of ASD cases. We suggest, that in the future, categorizing all putative susceptibility genes for ASD into biological pathways such as “synapse function” or “neuronal migration” or even more specific molecular signalling pathways such as those forming the basis of memory or learning will reveal general biological pathways that are abnormal in ASD patients. This will group ASD patients into biological meaningful entities that might require different treatments or genetic counselling strategies and might also identify new putative susceptibility genes (e.g.

protein binding partners). In addition, this strategy might be helpful in sorting irrelevant from relevant candidate susceptibility genes.

Since very little is known about most of the putative susceptibility genes for ASDs identified in our studies, functional studies, knock out models and association studies are needed to reveal which of these genes actually play a part in the development of ASDs. We are currently sequencing *KCNQ3* in 157 ASD patients and *NUDT6* and *FGF2* in 137 male ASD patients to shed light on their possible involvement in the aetiology of ASDs. However, due to the complex nature of ASDs a much larger cohort or additional functional studies will probably be needed to definitively weaken or affirm their role in the aetiology of ASDs.

10 APPENDIX A

Chr.	Band	Marker	LOD	Ref.
1	p13.2	D1S1675	2,15	[105]
	q23.1	D1S1653	2,63	[247]
	q42.2	D1S1656	3,06	[104]
2	q31.1	D2S2188	3,74	[103]
	q31.1-q31.3	D2S364-D2S335	2,39	[248]
3	q26.32	D3S3037	4,31	[247]
		D3S3715-D3S3037	4,81	[247]
4	q23	D4S1647	2,87	[104]
	q26.1	D4S3250	2,65	[104]
5	p13.1	D5S2494	2,18	[104]
		D5S2494	2,55	[102]
		D5S2494	2,54	[249]
	p14.3	D5S1473	2,13	[104]
6	q14..3	D6S1270	2,61	[104]
	q16.3	D6S283	2,23	[106]
7	q21.1	D7S1813	2,1	[250]
	q21.2	D7S1813	2,2	[251]
	q22.1	D7S477	3,2	[103]
	q32.2-q34	D7S530-D7S684	2,53	[108]
	q36.1	D7S483	2,13	[102]
	q36.2	D7S2462	3,04	[247]
10	p14	D10S1412	2,02	[104]
	q22.3	D10S2327	2	[104]
11	p13	D11S1392	2,1	[104]
		D11S1392	2,24	[249]
13	q12.2	D13S217-D13S1229	2,3	[251]
	q22.1	D13S800	2,54	[250]
		D13S800	3	[251]
16	p13.12-p13.13	D16S3102	2,93	[103]
	p13.13	D16S3102	2,93	[103]
17	q11.2	HTTINT2	2,34	[103]
		D17S1800	2,83	[249]
19	p13.12	D19S714	2,31	[104]
		D19S714	2,53	[102]
X	q11.1	DXS7132	2,75	[247]
	q25	DXS1047	2,67	[102]

Linkage result for ASDs with LOD \geq 2.0. Chromosome position refers to UCSC march 2006 (hg18).

11 APPENDIX B

Chr.	Band	Gene	Refs.
2	q33.3	NRP2	[76]
		GPR1	[76]
		ADAM23	[76]
		KLF7	[76]
		CREB1	[76]
		FZD5	[76]
	q34	MAP2	[76]
3		ERBB4	[76]
	q36.1	SCG2	[77]
	q27.3	SST	[78]
	q28	FGF12	[78]
	p12	GABRG1	[80]
	q21.21	FGF5	[78]
	q21.3	MAPK10	[78]
	q22.1	SNCA	[78]
	q22.1-22.1	GRID2	[78]
	q22.2	ATOH1	[78]
	q22.3	UNC5	[78]
	q27-28.1	FGF2	Paper III
	q28.1	NUDT6	Paper III
	q32.1	GLRB	[82]
		GRIA2	[82]
	q32.2	NPY1R	[82]
		NPY5R	[82]
	q34.1	GLRA3	[82]
	q35.2	FAT	Paper I
5	p14.1	CDH9	Paper IV
7	q11.22	AUTS2	[84]
	q11.23	FZD9	[78]
		STX1A	[78]
		LIMK1	[78]
		CYLN2	[78]
		GTF2IRD1	[78, 85]
		GTF2I	[78, 85]
	q22.1	REELIN	[86]
	q31.2	RAY1	[87]
8	q36.3	EN2	[88]
	q22.3	RIM2	Paper IV
		BAALC	Paper IV
	q24.44	KCNQ3	Paper II

Chromosome position and reference of candidate genes identified by chromosomal rearrangements.

Chr.	Band	Gene	Refs.
10	q22.3	KCNMA1	[89]
13	q13.2-13.3	NBEA	[92, 93]
	q13.3	MAB21L1	[92]
		DCAMKL1	[92]
		SMAD9	[92]
15	q11.2	NDN	[94]
		SNRPN	[94]
		SNURF	[94]
		UBE3A	[78, 94]
	q12	GABRB3	[78, 94]
		GABRA5	[78, 94]
18		GABRG3	[78, 94]
	q12.1	MAPRE2	Paper I
	q12.2	BRUNOL4	Paper I
		SLC39A6	Paper I
		ZNF397	Paper I
		ZNF396	Paper I
		ZNF271	Paper I
		ZNF24	Paper I
		STATIP1	Paper I
		GALNT1	Paper I
	q21.1	ZBTB7C	Paper IV
21	q22.2	PCP4	[78]
		DSCAM	[78]
X	p11.23	FTSJ1	[96]
		HDAC6	[96]
		PQBP1	[96]
		GRIPAP	[96]
		SYP	[96]
	p21.3	ARX	[36]
	p22.2	GRPR	[98]
	p22.31	VCX3A	[99]
	p22.31-22.32	NLGN4	[34, 99]

Chromosome position and reference of candidate genes identified by chromosomal rearrangements.

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